

**Insights into Prion Biology: Mechanistic Contributions and Phenotypic
Consequences of Oligopeptide Repeats**

By

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Providence Rhode Island, 2015

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This dissertation by Christine R. Langlois is accepted in its present form by the Department of Molecular and Cellular Biology and Biochemistry as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

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- Prion proteins can form self-templating, heritable aggregates in the cell and this ability is determined by specific sequence elements within the protein. Many prion proteins contain an oligopeptide repeat region, and a precise number of repeats is required in order to maintain the prion state. Using mutants that contain repeat deletions and expansions, I have proposed a model where the repeats affect the efficiency by which these protein complexes are processed by molecular chaperones.

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Chapter 1:
Introduction

Protein Folding and Quality Control

Protein folding in vitro

In order to be biologically active, most proteins must achieve a stable three-dimensional fold, either independently or upon their association with binding partners. Anfinsen demonstrated that all of the information necessary for a protein to fold *in vitro* resides in its sequence (Anfinsen, 1973). Indeed, when denatured *in vitro*, most proteins will spontaneously fold back to their native or near-native conformation upon removal of the denaturing agent. However, proteins cannot achieve their native conformations by random chance in a biologically relevant timeframe. As Levinthal postulated, the time it would take for a small 100-residue protein to fold by random sampling of all the possible conformations of its backbone residues alone would be around 10^{11} years. However, to fulfill their biological roles, proteins must be able to properly fold in a second or less (Zwanzig et al., 1992). Thus, to achieve their native conformation, proteins traverse folding pathways, where they adopt a series of partially folded intermediates in which only a small portion of the residues become ordered at a time, allowing the protein to bypass Levinthal's paradox. Smaller, model proteins follow what is known as the framework model, in which regions of secondary structure form first, and this is followed by collapse of these regions into a molten globule that closely resembles the native structure. Then, amino acid side chain interactions are formed until the final, native structure is reached (Ptitsyn, 1995). For larger proteins, the first step in this pathway is often

hydrophobic collapse, where the hydrophobic residues quickly collapse into a molten globule (Ptitsyn, 1995). The slow step in folding is then the reorganization of interactions within this globule. In these partially folded states, regions of the protein often have the correct secondary structure but lack the interactions necessary for the correct tertiary and quaternary structure. Interactions within the protein are then continuously remodeled until it reaches its most energetically favorable conformation (Dobson and Karplus, 1999).

Protein folding in vivo

While many proteins can spontaneously fold to the native state on their own *in vitro*, there are several challenges to protein folding in the context of the cell that are not present *in vitro*. First, it takes longer for a eukaryotic ribosome to synthesize a protein than for it to fold, which increases the chances for a protein to misfold before it has the necessary sequence information to arrive at its native conformation. Second, newly synthesized but not yet properly folded proteins are prone to aggregation via their exposed hydrophobic residues. Third, the cellular environment is extremely crowded, which increases the likelihood that a given protein will not fold correctly but instead will be recruited into aggregates (Frydman, 2001).

In order to overcome these challenges, the cell has evolved several chaperone networks to assist with protein folding. Small chaperones of the Heat Shock Protein (Hsp) 70 and Hsp40 families bind to exposed hydrophobic regions of nascent polypeptide chains, thereby preventing their aggregation (Pfund et al.,

1998; Yan et al., 1998). These chaperones stay bound until sufficient sequence is synthesized that the new polypeptide has the information it needs for folding to proceed (Hartl, 1996). Another class of HSPs, the small heat shock proteins (sHSPs) oligomerize into hollow, spherical structures, and bind unfolded polypeptide chains, providing a more conducive environment for proper protein folding (Haslbeck, 2002; Kim et al., 1998). In addition, larger chaperones sequester complete but unfolded polypeptides in a central cage, thereby preventing their aggregation, while at the same time providing an environment that is more conducive to proper folding (Hartl, 1996).

Protein misfolding in vivo

Despite the cellular networks in place to ensure proper folding, proteins in the cell often misfold as a result of mutations, stress or aging (Hartl and Hayer-Hartl, 2002). Misfolded proteins have an increased tendency to form aggregates, which can accumulate in the cell and lead to toxicity (Lansbury and Lashuel, 2006). Indeed, protein misfolding has been a major mechanism attributed to human diseases, including the neurodegenerative disorders Alzheimer's disease, Parkinson's disease, and Huntington's disease (Caughey and Lansbury, 2003). As such, the cell has evolved mechanisms to resolve misfolded protein species, ultimately leading to the misfolded species being either refolded or degraded.

In addition to their role in assisting the folding of newly synthesized proteins, chaperones also direct the fate of misfolded proteins. In one pathway, Hsp70 and Hsp40 chaperones cooperate with E3 ubiquitin ligases to mark

proteins for degradation (Cummings et al., 1998; Summers et al., 2013). Ubiquitinated proteins are then recognized by the 26S proteasome, which is the primary cellular machine responsible for regulated protein degradation in eukaryotes. The proteasome degrades misfolded soluble proteins, thereby preventing their aggregation. However, once protein aggregates form, the proteasome has limited capacity to act as a disaggregase (Cummings et al., 1998; Verhoef et al., 2002). In fact, aggregates may actually inhibit proteasome activity *in vivo*, due to the irreversible sequestration of proteasomes into aggregates (Navon and Goldberg, 2001; Verhoef et al., 2002).

In addition to functioning in degradative pathways, other chaperones exist to ensure the resolution of protein aggregates. In yeast, reactivation of aggregated proteins occurs via a bi-chaperone system composed of the Hsp70 Ssa1 and its co-chaperone Ydj1, and the AAA+ chaperone Hsp104 (Glover and Lindquist, 1998). A similar system exists in bacteria, where the homologs for Ssa1, Ydj1, and Hsp104 are DnaK, DnaJ, and ClpB, respectively (Mogk et al., 1999). Hsp70 and 40 chaperones first recognize misfolded, aggregated proteins via exposed hydrophobic residues or unstructured domains and bind these regions. Chaperone binding protects the aggregates from proteases, and allows for the transfer of aggregates to Hsp104 or ClpB, both of which assemble into hexamers, with individual subunits forming a ring around a central pore (Lee et al., 2003). After receiving the aggregates, Hsp104 or ClpB extract single polypeptides into the central pore via a threading activity (Lum et al., 2004). Substrate threading is mediated by aromatic residues inside the chaperone's

central pore, which interact with and pull the substrate through the central pore in an ATP-dependent manner (Lum et al., 2004; Schlieker et al., 2004). Evidence for the importance of the aromatic residues in threading comes from mutation studies in which conserved tyrosine residues were mutated to alanine. In these mutants, protein refolding is inhibited, without affecting other biochemical properties of the chaperone, including ATP hydrolysis. In addition, the local position of the aromatic residues in these conserved motifs is strongly influenced by the nucleotide binding state. In the absence of ATP or ADP binding, the aromatic residue is positioned close to the wall of the central channel, but in the presence of nucleotide binding, this residue moves, likely placing it in the center of the channel. This movement of the residues is hypothesized to serve as a “molecular ratchet” that pulls the substrate through the central channel (Lum et al., 2004). In addition to pulling substrates from an end, Hsp104 and ClpB can also thread internal segments through the central pore in a looped conformation (Haslberger et al., 2008). Substrate threading continues until it is complete or Hsp104 or ClpB encounters a stably folded domain, leading to substrate dissociation (Figure 1). The released polypeptide can then refold into a native conformation. Other AAA+ ATPase chaperones and proteases in bacteria, including ClpA, ClpC and ClpP have also been shown to have disaggregation activity and thread substrates through a similar mechanism (Tyedmers et al., 2010). Surprisingly, higher eukaryotes do not contain cytoplasmic homologs of Hsp104 or ClpB. However, mammalian cells are able to resolve aggregates, indicating the existence of an alternate pathway (Cohen et al., 2006; Lelouard et

al., 2002). Indeed, in mammals, Hsp110, a member of the Hsp70 family, works with the Hsp70/40 system to resolve protein aggregates *in vivo* (Rampelt et al., 2012). Hsp110 has both ATPase and nucleotide exchange activity, and only its NEF activity is required to solubilize protein aggregates *in vivo* and *in vitro*, suggesting that it regulates the ability of Hsp70 to hydrolyze ATP (Mattoo et al., 2013; Rampelt et al., 2012).

Bypassing the protein quality control machinery

In some cases, the generation of misfolded and aggregated proteins exceeds the capacity of the cell to refold or degrade them. For example, environmental stress, such as heat stress, leads to the bulk unfolding of a variety of cellular proteins, resulting in the formation of amorphous aggregates. To compensate for this increase in protein aggregation, the chaperones of the heat shock family are expressed at higher levels to resolve the abundance of aggregates that are produced (Gasch et al., 2000; Sanchez et al., 1992). In addition, mutations can also increase the propensity of particular proteins to misfold, as is the case in the proteins that cause Huntington's disease (huntingtin), as well as the familial forms of Alzheimer's (A β and tau) and Parkinson's disease (alpha-synuclein). In all of these diseases, the indicated misfolded proteins form amyloid aggregates, which are unable to be cleared by cellular machinery, leading to cell death and neurodegeneration (Chiti and Dobson, 2006; Ross and Poirier, 2004). In addition, another class of proteins, known as prion proteins, has the capacity to misfold and form β -sheet rich

amyloid aggregates. The aggregation of prion proteins changes their cellular activities, which can lead to cellular toxicity in mammals or new phenotypes in yeast (Cox, 1965; Griffith, 1967; Wickner, 1994).

The prion hypothesis

The prion hypothesis was originally proposed to explain the etiology of the transmissible spongiform encephalopathies (TSEs), a group of progressive neurodegenerative diseases in mammals that includes scrapie in sheep, bovine spongiform encephalopathy (mad cow disease) in cattle, and kuru and Creutzfeldt-Jacob Disease in humans (Prusiner, 1994). One of the first observations that linked this group of diseases was that brains of scrapie infected sheep and kuru patients had a similar spongiform pathology (Beck et al., 1966; Hadlow, 1959). In addition, brain homogenate isolated from scrapie-infected sheep could cause the disease to appear in healthy sheep and mice (CHANDLER, 1961). Similarly, kuru, which had become common among the Fore tribe in Papua New Guinea, where it was linked to the tribe's cannibalistic practices (Gajdusek and Gibbs, 1971), could be transferred to chimpanzees via brain homogenates (Gajdusek et al., 1966).

Prior to the study of the TSEs, heritable elements were thought to rely on DNA or RNA to encode and pass on their genetic information. However, methods that destroyed nucleic acids in tissue samples did not prevent inheritance of the disease, while methods that destroyed proteins rendered the

tissues inert, leading to the conclusion that the infectious agent was a *proteinaceous infectious particle*, or prion (Alper et al., 1967; Prusiner, 1982). The protein was later identified in mammals as the prion protein (PrP), and it was found that the normal cellular form of PrP (PrP^C, cellular) can adopt a protease-resistant alternate conformation (PrP^{Sc}, scrapie) (Bolton et al., 1982; McKinley et al., 1983). This idea that a protein could, on its own, be an infectious agent was originally controversial, however, an abundance of evidence has come to light in support of it, including the fact that purified PrP^{Sc} purified *in vitro* was sufficient to cause disease in mammals (Kim et al., 2010; Wang et al., 2010). In addition, the protein-only mode of inheritance is now known to be the mechanism underlying many inherited traits in yeast (Tuite and Serio, 2010).

Heritable information is traditionally thought to be derived from DNA, where the information is encoded in sequence. In contrast, the prion hypothesis states that a unique group of proteins can adopt alternate conformations, and once these conformations arise, they can template the conversion of like protein to the misfolded, or prion, state. Thus, once present, the alternate conformation confers a transmissible phenotype that can be faithfully propagated in a cell or whole organism (Griffith, 1967; Prusiner, 1982).

Prion Proteins in Yeast

In fungi, prions can serve as epigenetic elements of inheritance because they are transmissible through cell division and mating. Some of the best-studied

yeast prions include $[PSI^+]$, $[URE3]$, and $[PIN^+]$, which are determined by the Sup35, Ure2, and Rnq1 proteins, respectively (Cox, 1965; Sondheimer and Lindquist, 2000; Wickner, 1994). These proteins undergo a conformational switch to convert to the prion state, leading to the appearance of new phenotypes. For example, the normal function of the Ure2 is to repress transcription of genes needed to utilize poor nitrogen sources when rich nitrogen sources (i.e. ammonia) are present, and its conversion to the prion state ($[URE3]$) allows yeast to metabolize alternate nitrogen sources even in the presence of ammonia (Lacroute, 1971; Wickner, 1994). Similarly, the normal function of Sup35 is to catalyze polypeptide release during translation termination, and following its conversion to the prion state, Sup35 activity is reduced, leading to stop codon read-through (Cox, 1965; Ter-Avanesyan et al., 1994). This activity of Sup35 is advantageous in the laboratory. In a strain with a naturally occurring premature stop codon in the *ADE1* gene (*ade1-14*), strains in which Sup35 is in the non-prion ($[psi^-]$) state are unable to produce full-length Ade1 protein, and accumulate an intermediate in the adenine biosynthetic pathway, which gives the colonies a red color on rich medium (Figure 2A). In addition, because these strains cannot make adenine, they are unable to grow on media lacking adenine (Fisher, 1969). In its prion ($[PSI^+]$) state, however, Sup35 is unable to efficiently stop translation leading to stop codon read-through and production of full-length Ade1 protein. These colonies appear white on rich medium and are able to grow on medium lacking adenine (Figure 2B) (Chernoff et al., 1995; Cox, 1965; Patino et al., 1996; Paushkin et al., 1996). In contrast to

Ure2 and Sup35, Rnq1 has no known cellular function, but in its prion conformation $[PIN^+]/[RNQ^+]$, Rnq1 allows for the induction of other prions, including $[PSI^+]$ and $[URE3]$ (Derkatch et al., 1997, 2000, 2001).

Conformational Self-Replication

In order for prions to be heritable, there must be a mechanism for them to reliably self-replicate. The prion hypothesis predicts that the prion form of the protein binds to the properly folded form and stimulates the conformational conversion of the properly folded form to the prion state (Griffith, 1967). Consistent with this model, the non-prion form of mammalian prion protein, PrP^C , can be converted to prion, protease-resistant form, PrP^{Sc} , *in vitro*, in the presence of pre-existing PrP^{Sc} (Kocisko et al., 1994). The binding and conversion of PrP^C by PrP^{Sc} results in highly ordered aggregates, which must then be fragmented in order to provide new surfaces through which to template additional PrP^C to the prion form (Saborio et al., 2001). This mechanism has also been shown to apply to yeast prions, where Sup35 can spontaneously convert to the prion form and then template the conversion of non-prion Sup35 to the prion state. The addition of pre-existing Sup35 fibers and mechanical shearing of aggregates both enhance this reaction (Glover et al., 1997; Serio et al., 2000). In addition, purified Sup35 fibers made *in vitro* and transformed into non-prion $[psi^-]$ cells are sufficient to induce $[PSI^+]$ *in vivo* (Tanaka et al., 2005). Similarly, recombinant PrP^{Sc} fibers are sufficient to induce TSEs when inoculated into the brains of mice (Legname et al., 2004). Taken together, these data

indicate that existing protein in the prion form is both necessary and sufficient to convert non-prion protein to the prion state.

Prion Propagation in vivo

In addition to the conformational self-replication discussed above, there are additional cellular factors that are required for the prion state to persist *in vivo*. The prion propagation cycle requires three distinct and fundamental steps. First, soluble non-prion protein must be efficiently converted to the prion state. Second, these newly formed prion aggregates must be continually fragmented to ensure the availability of templates for conversion. Third, the prion aggregates must be efficiently inherited by daughter cells upon cell division (Sindi and Serio, 2009) (Figure 3).

Similar to the *in vitro* conversion reaction mentioned above, prion protein *in vivo* must adopt a self-templating alternative conformation, where existing prion aggregates bind to and remodel soluble non-prion protein, thereby converting it to the prion state. In mammalian cells, PrP transitions from a protease resistant form to a protease sensitive form within hours of being transfected with scrapie agent (Caughey and Raymond, 1991; Taraboulos et al., 1990). In yeast GFP-tagged soluble Sup35 is rapidly converted to the prion state when $[PSI^+]$ is introduced into the cell via mating (Satpute-Krishnan and Serio, 2005), indicating that the prion state can indeed be transmitted to existing non-prion protein.

Following the initial conversion step, prion aggregates must be fragmented, a critical step to ensure sufficient templates for conversion. The role of fragmentation was first shown to be important *in vitro*, where sample agitation or sonication accelerates fiber growth (Parsell et al., 1994; Serio et al., 2000). The mechanism of fragmentation for the mammalian prion is currently unknown, but in yeast, prion aggregates are fragmented by the molecular chaperone Hsp104 and its co-chaperones Ssa1 (Hsp70) and Sis1 (Hsp40) (Allen et al., 2005; Chernoff et al., 1995; Higurashi et al., 2008; Song et al., 2005; Tipton et al., 2008). Hsp104 is a member of the AAA+ ATPase family and is required for viability at elevated temperatures where its primary function is in the resolution of heat-denatured aggregates (Glover and Lindquist, 1998; Neuwald et al., 1999; Parsell et al., 1994). The direct role of Hsp104 in fragmentation has been implicated by several studies. First, deletion or inhibition of Hsp104 results in prion curing (Chernoff et al., 1995; Moriyama et al., 2000). Second, in [*PSI*⁺] cells with wild-type Hsp104 activity where prion aggregates are marked with Sup35-GFP, prion complexes become undetectable following inhibition of Sup35-GFP synthesis while untagged Sup35 is constitutively expressed. However, upon Hsp104 inhibition, the prion complexes persist, thus the loss of fluorescence in wild-type cells can be attributed to fragmentation activity, which redistributes the GFP-tagged Sup35 to a larger number of complexes which become less fluorescent through the addition of unmarked Sup35 (Satpute-Krishnan et al., 2007). Third, following inhibition of Hsp104 in an actively dividing culture, prion aggregates both increase in size and decrease in number, and reactivation of

Hsp104 is able to rescue both these defects (Eaglestone et al., 2000; Kryndushkin et al., 2003; Wegrzyn et al., 2001).

Lastly, in order for prion aggregates to remain heritable, they must be efficiently transmitted to daughter cells upon cell division. In yeast, fragmentation by Hsp104 is essential for this step. Upon inhibition of fragmentation, Sup35 aggregates become larger and less mobile, and these immobile complexes are not efficiently inherited following cell division, eventually leading to prion loss (Kawai-Noma et al., 2009; Satpute-Krishnan et al., 2007). Consistent with this observation, smaller aggregates have been shown to transmit more efficiently into daughter cells (Derdowski et al., 2010; Satpute-Krishnan et al., 2007). In mammals, prion disease occurs in neuronal cells, which are post-mitotic. However, throughout the course of the disease PrP^{Sc} must spread throughout the central nervous system, and it has been suggested that once prion aggregates are released into the extracellular space, they can diffuse into neighboring cells (Aguzzi and Rajendran, 2009; Beekes and McBride, 2007). In addition, non-infectious amyloids are also thought to spread within organisms through a similar mechanism (Brundin et al., 2010).

Structural Characteristics of Prion Proteins

Misfolding of proteins into both non-prion amyloids and prion aggregates is associated with an increase in β -sheet content. In their normal conformations, PrP has high α -helix content, while the prion domain of Sup35 is largely unstructured (Balbirnie et al., 2001; Pan et al., 1993). In their prion

conformations, however, both proteins propagate in an amyloid-like fashion, with high β -sheet content (Balbirnie et al., 2001; Pan et al., 1993). These amyloid-like fibers have increased protease resistance and can bind to amyloid-specific dyes such as Congo red (Glennner, 1980a, 1980b; Sipe and Cohen, 2000).

Due to the difficulty of studying the structure of amyloid fibrils using traditional methods, most structural studies of these fibers were done using short peptide segments, rather than full-length fibers. X-ray diffraction and NMR studies using a short Sup35 peptide revealed that these fibers have a predominantly cross- β structure, with the β -sheets running perpendicular to the fiber axis and hydrogen bonding between side chains of neighboring strands facilitated by glutamine and asparagine residues. In this structure, the position of each strand is slightly shifted compared to the neighboring strand, allowing the interdigitation of side chains, excluding water and forming a “steric zipper” (Nelson et al., 2005; van der Wel et al., 2007).

Sequence requirements to maintain alternative protein conformations

Role of Q/N-rich regions

For many amyloidogenic proteins, aggregation is mediated by a glutamine/asparagine (Q/N)-rich domain. In these proteins, high Q/N content is both necessary and sufficient to drive protein aggregation, but is not sufficient to modulate protein-based inheritance (Michelitsch and Weissman, 2000; Osherovich et al., 2004). The propensity of Q/N rich regions to aggregate may

be due to the ability of these side chains to form hydrogen-bonding networks, thereby mediating protein-protein interactions via “polar zippers.” Indeed, molecular modeling and structural data show that polyQ peptides form aggregates consisting of tightly linked β -sheets (Perutz et al., 1994).

For the yeast prion Sup35, the Q/N-rich region (amino acids 1-41) drives the conversion of soluble protein into the prion form (Osherovich et al., 2004). This region is conserved across all yeast species where Sup35 is capable of forming a prion, and is both necessary and sufficient to drive protein aggregation (Borchsenius et al., 2001; Santoso et al., 2000). Furthermore, mutation of Q/N residues within the prion-forming domain to polar or charged reduces the ability of soluble Sup35 to be incorporated into aggregates, resulting in increased levels of soluble Sup35 and a less severe prion phenotype, or in the most severe cases, prion loss (DePace et al., 1998; DiSalvo et al., 2011; King, 2001; McCready et al., 1977).

While it was originally thought that glutamines and asparagines played equivalent roles in prion formation (Michelitsch and Weissman, 2000; Osherovich et al., 2004), it was subsequently shown that the presence of asparagine promotes prion propagation to such an extent that aggregates with high N content can bypass cellular requirements for *de novo* prion formation, while the presence of glutamines decreased the formation of prion aggregates, and promoted the formation of toxic aggregates species (Halfmann et al., 2011; Ross and Toombs). This difference may be explained by the fact that asparagine has a shorter side chain, and thus polyasparagine tracts can more easily form

hydrogen bonded β -sheets and β -turns at the monomer level. The longer side chain of glutamine, however, prevents the formation of tight β -turns. Thus, polyglutamine β -sheet formation requires at least two interacting proteins which may promote non-specific interactions and off-pathway aggregation, leading to toxicity (Halfmann et al., 2011). In addition, the tendency of polyglutamine to form toxic species is particularly interesting, given the role of polyglutamine expansions in several neurodegenerative diseases.

Role of PolyQ repeats in cellular toxicity

At least a dozen human diseases are linked to expanded CAG repeats (polyglutamine), including Huntington's Disease, the spinocerebellar ataxias, and spinal-bulbar muscular atrophy. These diseases are all progressive neurological disorders that are inherited in an autosomal dominant manner. The proteins implicated in these diseases are not related, suggesting that there is a common pathogenic mechanism shared by all the diseases (Williams and Paulson, 2008). Several mechanisms have been proposed to explain polyQ toxicity, including misfolding of the disease-associated protein resulting in altered function, detrimental protein-protein interactions, and the formation of toxic oligomeric complexes (Williams and Paulson, 2008).

In these diseases, polyglutamine (polyQ) expansion leads to misfolding of the protein and its aggregation into cellular inclusions, which in turn leads to cytotoxicity (Bates, 2003; Zoghbi and Orr, 2000). For each disease, there exists

a threshold of polyQ repeat number, below which the protein is non-pathogenic, and above which, the disease phenotype arises. Furthermore, a larger expansion leads to more protein misfolding, an earlier disease onset, and faster disease progression (Andresen et al., 2007; Zoghbi and Orr, 2000), suggesting that expansion of the polyQ region promotes the conversion of soluble protein to the amyloid state.

Studies of polyQ aggregation and toxicity in yeast models have led to mechanistic insights. When GFP-tagged, N-terminal fragments of huntingtin protein (Htt) were expressed in yeast, their aggregation depended on the length of the polyQ repeats, with longer expansions resulting in an increase in quantity and intensity of foci (Krobitsch and Lindquist, 2000; Wang et al., 2009). In addition, this aggregation was dependent on the chaperone Hsp104, with overexpression of Hsp104 increasing the number of foci and deletion of Hsp104 abolishing the foci, suggesting that the aggregation and subsequent toxicity of polyQ proteins is dependent on chaperone systems within the cell (Krobitsch and Lindquist, 2000).

Interestingly, the toxicity of polyQ fragments can be mediated by the composition of flanking regions. Immediately adjacent to the polyQ tract in Htt, there is a 17 residue proline-rich region, and inclusion of this region in expanded polyQ tracts protects against cellular toxicity (Duennwald et al., 2006; Wang et al., 2009). Inclusion of the proline-rich region did not, however, mitigate protein misfolding, but rather induced the formation of non-toxic aggresomes (Duennwald et al., 2006). One explanation for this effect is that the polyQ and

poly-proline regions may affect the propensity of the polypeptide to form certain secondary structures. The polyQ region favors a β -sheet conformation, while poly-proline favors a proline-type-two helix, which antagonizes β -sheet formation (Duennwald, 2011). Consistent with this observation, polyQ proteins that do not adopt a β -sheet conformation have not been shown to be toxic to cells (Nagai et al., 2007).

Importance of Oligopeptide Repeats in Maintaining Alternative Conformations

In addition to the Q/N-rich regions, some amyloidogenic proteins contain oligopeptide repeats, which are necessary to maintain their alternative conformation. An aggregation-prone sequence (i.e. polyQ) becomes a heritable element when fused to an oligopeptide repeat region (Osherovich et al., 2004). The mammalian PrP contains five copies of an octarepeat, and Sup35 contains five and a half copies of a nonarepeat (Figure 4). For both proteins, deletion of the oligopeptide-repeat region compromises prion propagation, while expansion of the oligopeptide-repeat region increases the spontaneous appearance of the prion form (Liu and Lindquist, 1999; Parham et al., 2001; van Rhee et al., 2003). Oligopeptide-repeat regions are also present in the yeast prions [*NEW1*] and [*RNQ*], as well as in the bacterial amyloidogenic protein CsgA (Cherny et al., 2005; Osherovich et al., 2004; Vitrenko et al., 2007). However, the yeast prion [*URE3*] does not contain oligopeptide repeats (Maddelein and Wickner, 1999), indicating that the role of the oligopeptide repeat region is limited to specific proteins.

Interestingly, the sequence of the oligopeptide repeats in Sup35 can be scrambled while keeping the amino acid composition intact, and these mutants are able to support $[PSI^+]$, suggesting that primary sequence composition and not the sequence of amino acids in the repeats *per se* is the necessary determinant. However, these scrambled mutants are incompatible with $[PSI^+]$ propagated by wild-type Sup35 and show high levels of prion loss on their own, indicating that the presence of repeats, and not just primary sequence, plays an important role (Ross et al., 2005; Toombs et al., 2011).

Effects of Octarepeat region in PrP

The mammalian prion protein (PrP) contains five copies of an octapeptide repeat with the consensus sequence PHGGGWGQ (Kretzschmar et al., 1986). Cases of inherited prion disease have been described for families containing an insertion of five (Jansen et al., 2009), six (Mead et al., 2006), or seven (Jansen et al., 2011; Mauro et al., 2008) extra repeats. In addition, isolated cases of prion disease have been reported for individuals harboring between one and nine extra repeats (Castilla et al., 2004; Guo et al., 2008; Martin et al., 2009; Mauro et al., 2008; Yanagihara et al., 2002). These individuals generally showed an earlier age of disease onset and faster disease progression when compared to wild type individuals who developed prion disease sporadically (Goldfarb et al., 1991; Yanagihara et al., 2002).

To investigate the mechanism by which repeat expansions lead to prion disease, model systems have been developed in mice. Mice expressing PrP

completely devoid of the repeat region are still susceptible to prion disease following inoculation with prion-infected brain homogenate, but these mice have a longer incubation time to disease, and lower levels of proteinase K resistant PrP in the brain but not in the spinal cord, suggesting that the octarepeat region may play a role in conversion specifically in the brain (Flechsigs et al., 2000; Yamaguchi et al., 2012). In addition, mice containing only one intact repeat do not show longer incubation times following prion inoculation, but these animals still show lower levels of prion infectivity in the brain (Yamaguchi et al., 2012). In contrast, mice expressing PrP containing a one-repeat insertion show reduced incubation times to disease and higher prion titers earlier in life (Castilla et al., 2004). Similarly, mice containing PrP with fourteen repeats show faster disease progression and detectable levels of proteinase K resistant PrP as early as the first week of life (Chiesa et al., 2000). Taken together, these data suggest that the repeat region is not required for PrP aggregation or disease progression, but it enhances the ability of prion aggregates to propagate once present.

The repeat region in PrP has been shown to coordinate copper binding via its histidine residues (Millhauser, 2004), and there are conflicting reports on how disease progression may be influenced by copper binding. In the first model, copper binding enhances the ability of PrP to convert to the prion state, and PrP containing repeat insertions bind more copper and thus promote faster disease progression (Yu et al., 2008). Consistent with this idea, treatment of scrapie-inoculated mice with a copper chelator delays onset of disease (Sigurdsson et al., 2003). However, expansion of the repeat region to sixteen repeats has been

shown to allow PrP^{C} monomers to bind PrP^{Sc} in a copper-independent manner, thereby accelerating conversion to the prion state (Leliveld et al., 2006). In the second model, the repeat expansion leads to changes in copper occupancy, and increases the affinity of the repeat region to bind copper. Copper can bind PrP repeats with different occupancies: in the low occupancy state, the entire octapeptide region is wrapped around a single copper ion, whereas in the high occupancy state each repeat coordinates the binding of a single copper ion (Chattopadhyay et al., 2005). PrP monomers with eight or nine repeats require higher equivalents of copper in order to switch from the low to high occupancy states compared to PrP monomers containing seven or fewer repeats. Interestingly, this change in copper binding correlates with age of disease onset in humans, where individuals with nine repeats show an almost three decade decrease in average age of onset, compared to those with seven repeats (Stevens et al., 2009). This correlation can be explained by the fact that copper has been shown to inhibit the conversion of PrP to the amyloid state (Bocharova et al., 2005), thus in this model the repeat expansion binds less copper than WT and is therefore more easily able to convert to the prion state (Stevens et al., 2009).

Other studies have investigated the role of the PrP octarepeat region by introducing this region into the yeast prion protein, Sup35 in place of repeats 2-5.5. In this chimeric system, increasing repeat number resulted in an increased conversion to the $[\text{PSI}^+]$ state. However, further expansion also resulted in an increased prion loss, suggesting that repeat expansion destabilizes both the

[PRION+] and [prion-] states (Dong et al., 2007). In addition, overexpression of the chaperone Hsp104 stabilizes the [PRION+] state in a Sup35 chimera containing 14 PrP repeats (Tank et al., 2007), suggesting that these chimeras require higher levels of fragmentation to be faithfully propagated. Consistent with these *in vivo* studies, *in vitro* conversion rates correlate directly with repeat number, and these fibers do not differ in their thermodynamic stabilities, suggesting that these chimeric fibers do not differ in their physical properties. Interestingly, Sup35 fibers containing 14 PrP repeats are able to cross seed with both WT Sup35 and Sup35 containing 5 PrP repeats, suggesting that cross-seeding may be a factor in disease progression, where most individuals are heterozygotes (Kalastavadi and True, 2008).

Effect of Repeat Deletions and Expansions for Sup35

Sup35 contains five and a half imperfect oligopeptide repeats with the consensus sequence PQGGYQQYN, beginning at amino acid position 42 and ending at 97 (Kushnirov et al., 1988). Deletions or mutations within this region have been shown to compromise prion propagation, while expansions have been shown to increase the appearance of the prion state (Liu and Lindquist, 1999; Osherovich et al., 2004; Parham et al., 2001; Shkundina et al., 2006). The presence of all 5.5 repeats is required in order to fully maintain the $[PSI^+]$ phenotype. When just the last half repeat is deleted, cells are still able to maintain $[PSI^+]$. However these strains show increases in both translation

termination efficiency and levels of soluble Sup35, suggesting that prion propagation is still compromised (Osherovich et al., 2004).

Sup35 monomers deleted for the first two repeats, or for repeats 2-5 (RΔ2-5) are not able to induce $[PSI^+]$ *in vivo*, either at endogenous levels or upon overexpression. Furthermore soluble Sup35 containing either of these deletions is not able to co-aggregate with wild-type Sup35 (Liu and Lindquist, 1999; Osherovich et al., 2004). In addition, RΔ2-5 monomers are able to spontaneously form amyloid fibers *in vitro*, although this reaction occurs much more slowly than that of WT Sup35, suggesting that this region may be influencing the ability of the unfolded state to convert to a more highly ordered structure (Liu and Lindquist, 1999). However, it has also been reported that RΔ2-5 does not form amyloid-like fibers, but rather forms off-pathway filaments. The filaments show a higher random coil structure, when compared to β -sheet rich fibers, and do not show insensitivity to protease digestion or SDS. In addition, these filaments are stable on their own but cannot be converted to amyloid-like fibers capable of seeding a conversion reaction (Hess et al., 2007). Taken together, these data suggest that Sup35 monomers containing internal repeat deletions cannot propagate $[PSI^+]$ on their own, and are incompatible with wildtype $[PSI^+]$ aggregates.

In contrast to examining the effect of internal deletions, other studies have examined the effects of progressive repeat deletions from the C terminal end of the repeat region. The repeat deletions used in these studies also contained a deletion of a fourteen amino acid segment immediately C-terminal to the repeat

region (amino acids 98-111), believed to have no effect on prion propagation (Parham et al., 2001). These deletion studies showed that at least the first five full repeats were required to maintain the $[PSI^+]$ state when the deletion was the only copy of Sup35 present in the cell. In the presence of WT Sup35 only the first two repeats were required to induce and maintain $[PSI^+]$, suggesting that these first two repeats may affect the ability of Sup35 monomers to efficiently convert to the prion state (Osherovich et al., 2004; Parham et al., 2001). However, another group reported that $[PSI^+]$ could be maintained in cells with only the first four repeats when expressed as the only copy, and that zero repeats were required when expressed in combination with wild type, although in both cases these strains had an increase in prion loss (Shkundina et al., 2006). The discrepancy in the minimum number of repeats required may be due to differences in the prion variant used or in yeast strain background.

At the biochemical level, deletion of repeats results in an increase in the size of prion aggregates, both when the deletions are expressed on their own or in combination with WT, suggesting that aggregates containing repeat deletions may not be efficiently recognized or processed by the chaperone, Hsp104 (Shkundina et al., 2006). Consistent with this idea, deletion mutants missing the first two repeats ($\Delta 22-69$), which can support $[PSI^+]$ under selective conditions but show a high degree of prion loss under non-selective conditions, can be stabilized by increased levels of Hsp104 (Borchsenius et al., 2001).

In contrast to repeat deletions, yeast strains that contain Sup35 with two extra copies of the second repeat (R2E2) show an increase in the spontaneous

appearance of $[PSI^+]$, as well as an increase in the ability to induce $[PSI^+]$ upon overexpression. *In vitro*, the R2E2 mutant can assemble fibers much more rapidly than WT, in both seeded and unseeded reactions (Hess et al., 2007; Liu and Lindquist, 1999), suggesting that R2E2 Sup35 monomers have an increased propensity to convert to prion fibers..

In addition to the *in vivo* and *in vitro* studies mentioned above, an *in silico* approach has previously been used to model the prion pathway dynamics of various Sup35 mutants. This study utilized a two-step model of nucleation (conversion to the prion state) followed by autocatalytic growth (fragmentation of aggregates). No significant difference in nucleation was observed with different repeat numbers, however this model predicts an increase in autocatalytic growth with increasing repeat numbers (Liu and Lindquist, 1999; Watzky et al., 2008). This data is consistent with previous data in which the first 40 amino acids of Sup35 drive *in vivo* conversion, while the oligopeptide repeat region is largely responsible for faithful propagation of the prion form (Osherovich et al., 2004).

Conservation of repeats in functional amyloids

While much work has been done on the importance of polyQ and oligopeptide repeats in disease and prion propagation, repeats are also important for the formation of functional amyloids. The best characterized example of this effect is that of the curli protein, CsgA, in *E. coli*, where self-assembly of CsgA into amyloid facilitates biofilm formation (Chapman et al., 2002). The CsgA protein contains five copies of a hexapeptide repeat, with the sequence

QFGGGN (Figure 4). This sequence shows striking similarity to the PrP and Sup35 repeats, all of which contain Gln/Asn residues, aromatic residues, and multiple glycine residues, and it has been suggested that this combination of residues is important for amyloid assembly, whereby hydrophobic interactions facilitated by Gln/Asn residues, aromatic interactions, and the flexibility provided by the glycine residues lead to increased rate of auto-catalytic growth (Cherny et al., 2005). In addition, the CsgA repeats have been shown to bind copper with similar stoichiometry to that of PrP, and *E. coli* grown in increasing copper concentrations give rise to denser biofilms (Cherny et al., 2005), consistent with a model in which copper binding promotes conversion to the amyloid state (Sigurdsson et al., 2003; Yu et al., 2007).

Evidence for selection of optimal repeat number

All placental mammals have between two and seven repeats in the prion protein, with most orders having between five and six (van Rheede et al., 2003). Since copper binding to the repeat region is important for proper PrP function as well as maintaining cellular homeostasis, a sufficient number of repeats must be present. While it is not clear how many repeats must be present, the shortest reported number of repeats occurs in goats, where an allele with only three repeats is common (Goldmann et al., 1998), and alleles with only two repeats have been reported in lemur and squirrel, suggesting that this low number is evolutionarily viable (van Rheede et al., 2003). However, an increase in repeat

number leads to disease onset. Thus, selection of repeat number in mammals is driven by a competition for maintaining copper homeostasis, while at the same time avoiding disease (van Rheede et al., 2003).

Similarly, for yeast prions, all yeast species in which Sup35 is capable of forming a prion harbor between five and six repeats (Nakayashiki et al., 2001; Parham et al., 2001; Santoso et al., 2000). In contrast, Sup35 in the yeast *S. pombe* does not contain repeats, and consistent with this omission, is unable to form a prion (Ito et al., 1998; Parham et al., 2001). Because deletion of repeats in yeast prevents proper maintenance of the $[PSI^+]$ state and expansion of the repeat region does not allow the $[psi^-]$ state to persist, it has been suggested that selection of repeat number in yeast has evolved in order to efficiently maintain both the $[PSI^+]$ and $[psi^-]$ states with minimal switching (Parham et al., 2001).

How do the repeats contribute mechanistically to prion propagation?

Together, the studies discussed above suggest that repeats are not important for the initial conversion to the prion state, but are required for the efficient propagation of the prion form once it arises, but the precise mechanism by which the repeats act is unknown. In yeast, prion propagation requires both the continued incorporation of soluble protein into existing aggregates, and the fragmentation of these aggregates by molecular chaperones (Chernoff et al., 1995; Sindi and Serio, 2009). Furthermore, fragmentation can be divided into two events: prion aggregates must first be recognized and bound by Hsp104, and then a monomer must be extracted from the aggregate by threading through

the enzyme's central pore (Lum et al., 2004; Tyedmers et al., 2010). Here, we dissect the role of the repeat region of the Sup35/[PSI⁺] prion to determine its mechanistic contribution to prion propagation.

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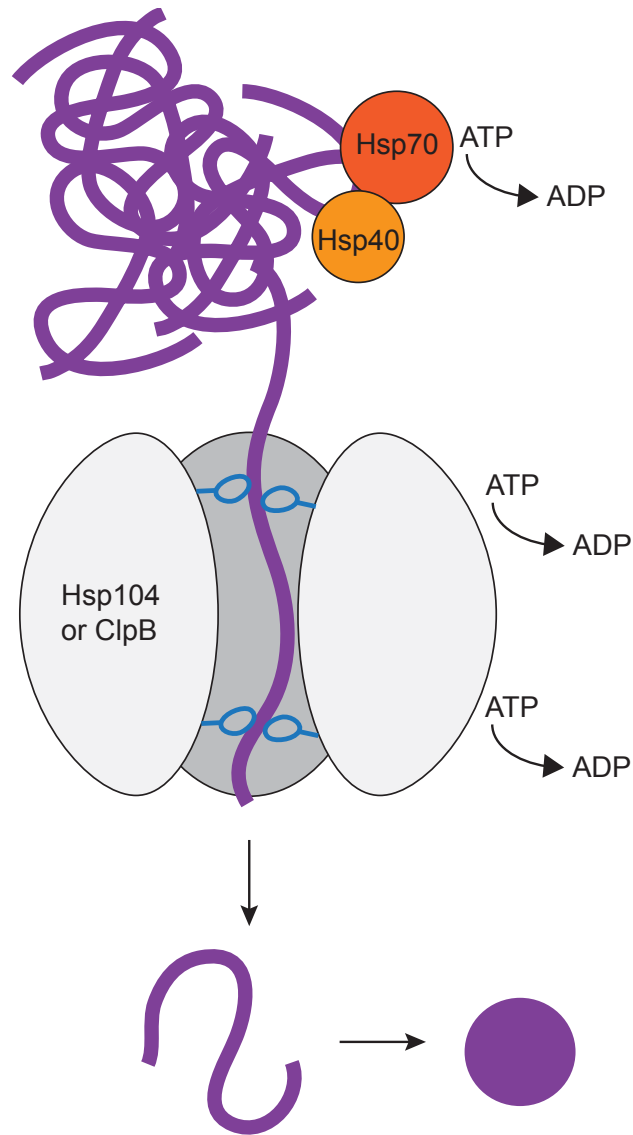


Figure 1. Resolution of aggregates by the AAA+ chaperones Hsp104 and ClpB. Misfolded and aggregated proteins are recognized by Hsp70 in cooperation with Hsp40 and transferred to Hsp104 or ClpB, which removes polypeptides and threads them through a central channel. Threading is mediated by conserved aromatic residues (blue loops), and ATP binding and hydrolysis, which induces conformational changes in the aromatic residues, thereby generating a force that pulls the substrate through the channel. Following substrate release, the polypeptide chain folds into the native conformation (purple circle).

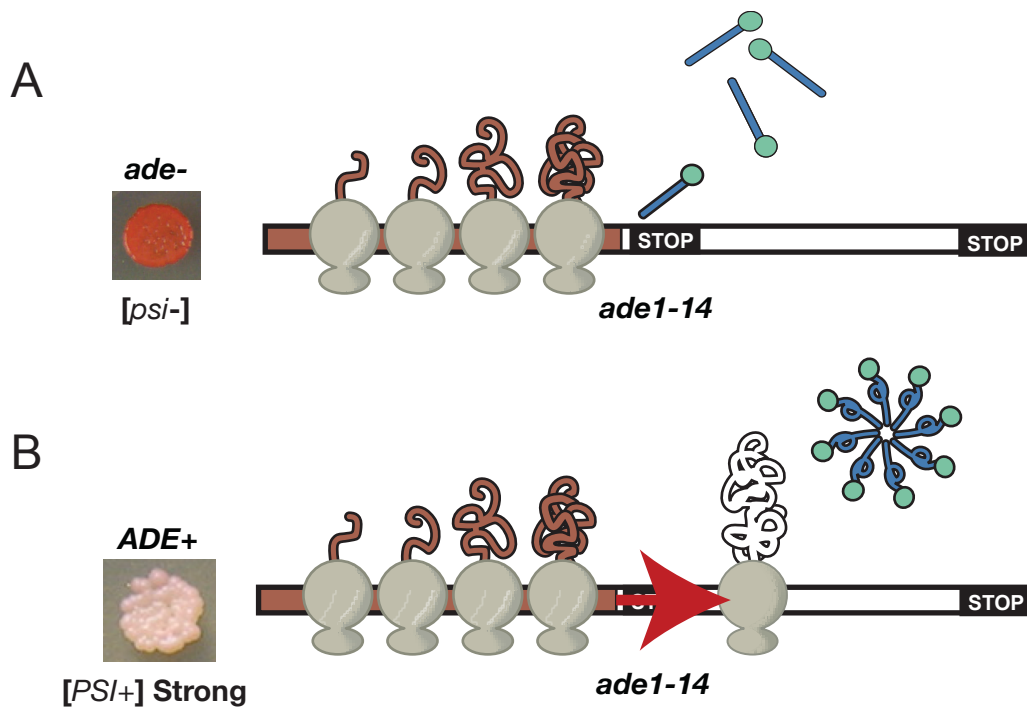


Figure 2. Sup35/[*PSI*⁺] Prion Phenotypes.

(A) In non-prion ([*psi*⁻]) cells, soluble Sup35 is able to efficiently stop translation at a premature stop codon in the *ade1-14* gene, leading to the production of a truncated product, the build up of an precursor in the adenine biosynthetic pathway and a red colony color. (B) In prion ([*PSI*⁺]) cells, Sup35 aggregates and is not available to stop translation, leading to production of the full length-product and a white colony color.

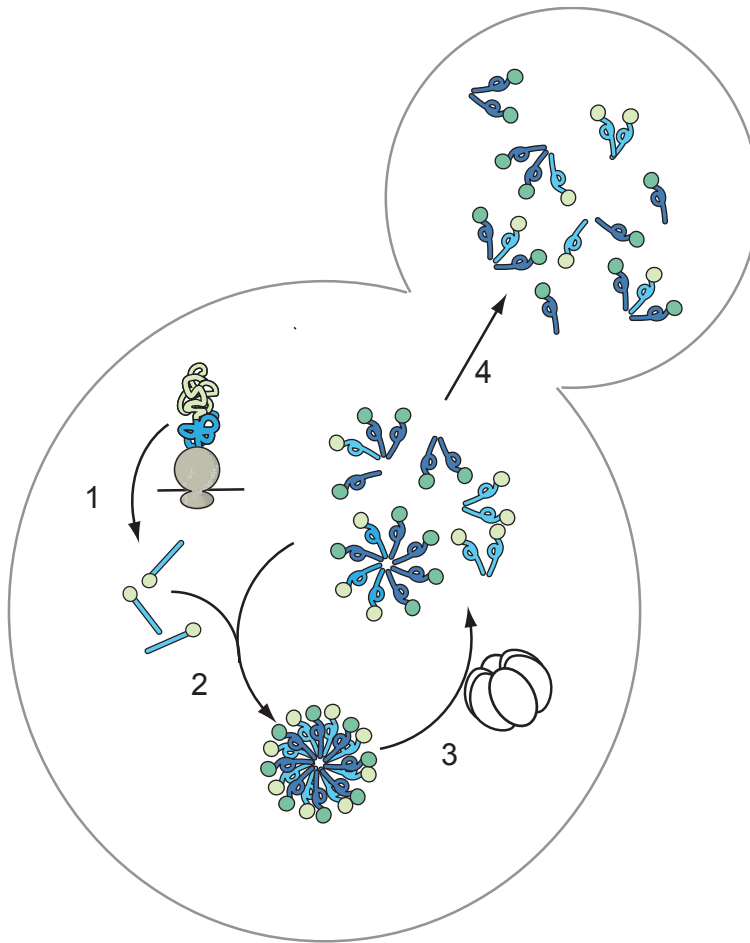


Figure 3. Prion Propagation in Yeast. Following synthesis (1), Sup35 must join existing aggregates and be converted to the prion form (2). These aggregates are then fragmented by the molecular chaperone Hsp104 (3), a step which both creates new templates for conversion and facilitates in the transmission of aggregates to daughter cells (4).

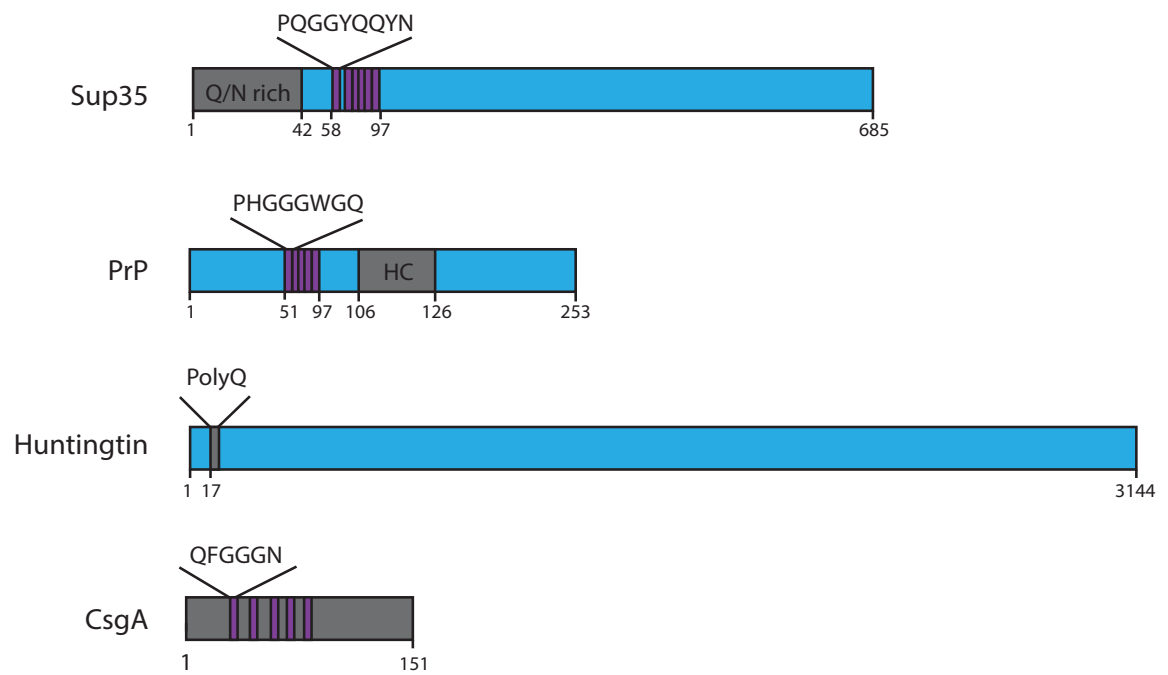


Figure 4. Conserved domains of amyloid proteins. Repeat sequences are shown in purple, and aggregation prone domains are shown in gray.

Chapter 2:
**The Oligopeptide Repeat Region Modulates Prion Propagation by Altering
Processing by Molecular Chaperones**

This chapter is in preparation for submission as a manuscript.

I performed all of the experiments in this chapter.

Abstract

Prion proteins can adopt multiple distinct and stable conformations: a properly folded form and a collection of misfolded or prion forms that are each associated with a unique phenotype. The prion phenotype is stabilized through a multi-step process that is dictated by specific sequence elements within the prion protein. First, protein in the non-prion form must be efficiently converted to the prion state. Second, the resulting aggregates must be efficiently fragmented by molecular chaperones. Many prion proteins contain oligopeptide repeats, the deletion of which abolishes the prion form, and expansion of which increases the spontaneous appearance of the prion. However, the precise role of these repeat regions in prion propagation has not been determined. Here, we use the Sup35/[PSI⁺] prion as a model to determine the mechanism by which the oligopeptide repeat region modulates prion propagation. We show that repeat expansion results in increased prion aggregate fragmentation, and that repeat deletions, in combination with a deletion of a downstream element, inhibit prion aggregate fragmentation. In addition, deletion or expansion of these elements do not affect the physical properties of the prion aggregates themselves, but rather affect the interactions of the prion aggregates with molecular chaperones.

Introduction

The misfolding of otherwise normal, cellular proteins has been implicated in a variety of neurodegenerative diseases, including Alzheimer's, Huntington's and Parkinson's diseases (Chiti and Dobson, 2006). The toxicity of these misfolded proteins is inherently linked to their ability to assemble into ordered, β -sheet-rich, linear aggregates, known as amyloids. Intriguingly, the ends of amyloid aggregates serve as templates to promote the misfolding of normal conformers of the same protein, which adopt the amyloid state by interacting with these surfaces and incorporating into the complex (Griffith, 1967). This self-perpetuation is responsible for both the persistence and amplification of amyloid *in vivo* and the associated progressive degeneration and invariant fatality of amyloid-associated diseases (amyloidoses) (Chiti and Dobson, 2006).

While the amyloid structure is driven by backbone-derived hydrogen bonding and likely represents a vestigial structure (Chiti and Dobson, 2006), the propensity of proteins to form these structures under physiological conditions is dependent on their sequence composition. First, a glutamine/asparagine (Q/N)-rich domain often mediates aggregation, and the propensity of Q/N-rich regions to aggregate is thought to be due to their propensity to form hydrogen bonding networks, making these residues particularly prone to mediate protein-protein interactions via "polar zippers" (Perutz et al., 1994; Sunde and Blake, 1997). Second, amyloidogenic regions are depleted for prolines and charged amino acids, which are thought to act as amyloid breakers (López de la Paz and

Serrano, 2004). Finally, beyond overall amino acid content, amyloid formation is sensitive to the position of specific amino acids, with the spacing of these amyloid breaking residues being particularly important (Alberti et al., 2009; López de la Paz and Serrano, 2004). But, while these sequence features clearly mediate the ability to form amyloid *in vitro*, our knowledge of the mechanisms by which these features promote amyloid persistence *in vivo* is currently unclear.

Prion proteins are a unique class of amyloids, which cannot only adopt alternative conformations (i.e. prion or amyloid and non-prion or normal), but also transmit these complexes to other cells or organisms as heritable or infectious elements (Bolton et al., 1982; Griffith, 1967; Serio et al., 2000). The appearance, persistence and transmissibility of prion amyloid aggregates is based on the presence of a prion-forming domain (PrD), which is both necessary and sufficient for all aspects of prion biology. In addition, PrDs are modular and can be fused to other proteins to create novel self-replicating aggregates (Li and Lindquist, 2000). Like other amyloids, PrDs have a unique sequence composition, as they are enriched for glutamines, asparagines, tyrosines and glycines, and this amino acid composition supports the conversion of soluble proteins into β -sheet-rich amyloid, and subsequently, the propagation and inheritance of these aggregates (Alberti et al., 2009; Alexandrov et al., 2012, 2008; Serio et al., 2000).

Here, we use the Sup35/[*PSI*⁺] prion protein in *S. cerevisiae* as a model system to study the requirements of specific sequence elements for amyloid formation. Sup35 is an essential component of the translation termination

machinery, and its conversion to the prion ($[PSI^+]$) form leads to stop codon read-through (Cox, 1965; Ter-Avanesyan et al., 1994). In a strain containing a naturally occurring premature stop codon in the *ADE1* gene (*ade1-14*), Sup35 in the non-prion ($[psi^-]$) form leads to efficient termination and the buildup of a precursor in the adenine biosynthetic pathway, which results in yeast colonies that are red on rich medium and unable to grow on medium lacking adenine. In its prion form, Sup35 misfolds and aggregates, leading to translation termination read-through, and this event creates yeast colonies that are white on rich medium and able to grow on media lacking adenine (Cox, 1965; Patino et al., 1996; Paushkin et al., 1997). This clear and protein-conformation based phenotype makes the Sup35/ $[PSI^+]$ prion an experimentally tractable model to dissect the contributions of PrD sequence to amyloid appearance, persistence and transmissibility *in vivo*.

The Sup35/ $[PSI^+]$ prion protein contains a bipartite N-terminal PrD containing a Q/N-rich region (amino acids 1-41) followed by five and a half imperfect oligopeptide repeats (amino acids 42-97), the consensus sequence of which is PQGGYGGYN (Kushnirov et al., 1988). Protein-protein interaction in prion aggregates is mediated by the Q/N-rich region, which is required to drive the conversion of soluble protein to the prion form (Osherovich et al., 2004), and mutations in this region reduce the ability of Sup35 to incorporate into aggregates (Chien and Weissman, 2001; King, 2001; McCready et al., 1977). However, the Q/N-rich region on its own is not sufficient to mediate protein-based inheritance, which also requires the presence of the oligopeptide repeat region (Michelitsch

and Weissman, 2000; Osherovich et al., 2004). Sup35 lacking the entire repeat region is not able to induce $[PSI^+]$ upon its overexpression or join onto existing wildtype aggregates. When full-length Sup35 is present, only one oligopeptide repeat is required for $[PSI^+]$ induction and incorporation of Sup35 into existing aggregates. However, in the absence of full-length Sup35, at least five oligopeptide repeats are required for prion maintenance (Osherovich et al., 2004; Parham et al., 2001; Shkundina et al., 2006). In contrast, expansion of the repeat region increases the spontaneous appearance of $[PSI^+]$ *in vivo* and results in faster conversion *in vitro* (Hess et al., 2007; Liu and Lindquist, 1999). Interestingly, an aggregation-prone sequence (i.e. polyQ) can be turned into a heritable element when fused to the oligopeptide repeats (DePace et al., 1998; Osherovich et al., 2004), highlighting the importance of the oligopeptide repeat region for the stable propagation of protein epigenetic elements. Indeed, this requirement for such a bipartite prion domain is not unique to $[PSI^+]$. Other yeast prions (i.e. New1) as well as the mammalian prion protein (PrP) show similar requirements (Goldfarb et al., 1991; Maddelein and Wickner, 1999; Osherovich et al., 2004). However, the exact mechanism through which the repeats act to affect prion propagation *in vivo* remains unclear.

The fact that repeats are not required to join existing aggregates, but are required for prion maintenance, suggests that the repeats influence the efficiency of at least one step of the prion propagation cycle *in vivo*: conversion, fragmentation, or transmission. Following an initial nucleation event, newly made, soluble protein must first be able to join prion aggregates, thereby

converting to the prion state (Satpute-Krishnan and Serio, 2005). Next, in order to remain heritable, these aggregates are fragmented by the molecular chaperone, Hsp104 (Chernoff et al., 1995). Hsp104 is a barrel-shaped, hexameric AAA+ ATPase, which acts on aggregated substrates by extracting single polypeptides and threading them through its central pore (Lum et al., 2004), and this action is thought to lead to the fragmentation of prion aggregates. Fragmentation is critically important for the inheritance of prion aggregates as it both creates new templates for continued conversion and ensures that aggregates remain small enough to be efficiently transmitted to daughter cells (Derdowski et al., 2010; Satpute-Krishnan et al., 2007; Serio et al., 2000).

Here, we identify the mechanism through which the repeat region affects prion propagation. Our results suggest that the repeats act at the fragmentation step. In addition, we have identified a new sequence element immediately C terminal to the repeats (the Repeat Proximal Region, RPR), which modulates the effect of the repeats. Importantly, the repeat and RPR region does not affect the physical properties of the prion aggregates themselves, but rather affects the efficiency by which molecular chaperones are able to act on prion aggregates, leading to their fragmentation.

Results

Repeats impact the severity of the [PSI⁺] phenotype and are modulated by a newly identified sequence element

To determine the mechanistic contribution of the repeat region to prion propagation, we constructed a series of repeat variants (Figure 1A), each composed of full-length Sup35 containing a different number of repeats. This collection includes repeat deletion strains (R1-X), which contain the N terminal X repeats, from 2 to 5, and repeat expansion strains (R2E1 and R2E2), which contain one or two extra copies of the second repeat for a total of 6.5 or 7.5 repeats, respectively. In contrast to previous repeat variant studies, which overexpressed the Sup35 protein from a plasmid, we conducted our studies using strains in which the repeat variants were all expressed from integrated constructs at levels similar to wildtype (Figure S1) to control for the fact that protein expression levels affect the dynamics of prion propagation (Allen et al., 2005; Derdowski et al., 2010; Sindi and Serio, 2009). Each repeat variant is expressed from its own promoter at the endogenous locus. In constructing these strains, we noticed that the repeat expansion strains (R2E1 and R2E2), expressed Sup35 at a much lower level than either wildtype or the repeat deletion strains. To ensure that Sup35 was expressed at the same level across the entire repeat variant collection, we integrated a second copy of Sup35 with the repeat expansion at another locus in these strains (see methods).

We first examined the prion phenotypes of strains in our repeat variant collection to confirm the number of repeats required to support [*PSI*⁺]. The phenotypes of strains containing four, five, or wildtype repeat number were phenotypically indistinguishable (Figure 1B, left), but the strain expressing R1-4 lost the [*PSI*⁺] prion at a frequency of ~3% in comparison to the other strains, in

which [*PSI*⁺] was fully stable. When the number of repeats was expanded, the colonies appeared more white on rich medium and showed slightly better growth on medium lacking adenine, suggesting higher levels of stop codon read-through in these strains. Repeat deletion strains containing only 2 or 3 repeats (R1-2 and R1-3, respectively) were unable to support [*PSI*⁺], indicating that at least four repeats are required for prion maintenance.

Previous studies have reported that at least five repeats are required for [*PSI*⁺] maintenance. In exploring the basis of this difference from our observations, we noticed that the previous studies on repeat deletions also deleted a fourteen amino acid region immediately C-terminal to the repeats (Osherovich et al., 2004; Parham et al., 2001; Shkundina et al., 2006), which we have termed the Repeat Proximal Region or RPR. The sequence of the RPR is RGNYKNFNYYNNLQ, and because of the Q/N rich nature of this region, we hypothesized that it might modulate the effect of the repeat deletion. To test this hypothesis, we constructed strains containing 4, 5 or wildtype repeats that also contain an RPR deletion (R1-4 Δ RPR, R1-5 Δ RPR, and Δ RPR, respectively). Consistent with previous studies, when the RPR was deleted, at least 5 repeats were required to maintain [*PSI*⁺] (Figure 1B, right). In all three strains, those lacking the RPR were more pink on rich medium and grew less well on medium lacking adenine compared to a strain containing the RPR and the same number of repeats (Figure 1B, right), suggesting that loss of the RPR increases the severity of the effect of repeat deletion alone.

The repeats and RPR act at the fragmentation step of prion propagation

The changes observed in the phenotypes of the repeat variants suggests that at least one step of the prion propagation cycle is being altered by their presence. To identify these defects, we characterized a representative subset of the repeat variant strains: R1-5, R2E1, Δ RPR. For the repeat deletions and expansions, we chose the most conservative changes because deletion or expansion of an additional repeat (i.e. R1-4 and R2E2) led to significant toxicity in the presence of $[PSI^+]$ (see Chapter 3).

Because the repeats have previously been shown to be important for maintenance of the prion form but not the addition of non-prion protein onto aggregates (Osherovich et al., 2004), we wondered if the repeats might affect the *in vivo* amplification of prion aggregates. To ask this question, strains were grown in rich medium containing guanidine HCl (GdnHCl), which reversibly inhibits Hsp104 (Ness et al., 2002), to reduce Sup35 aggregate number to near curing. Strains were then allowed to recover in rich medium in the absence of GdnHCl, to allow reamplification of aggregates (Tanaka et al., 2006). Following release from GdnHCl, individual cells were isolated at various timepoints by micromanipulation on rich medium containing GdnHCl, and the number of heritable units (propagons) per cell was determined by dispersing the resulting individual colonies on rich medium lacking GdnHCl. During this final GdnHCl step, existing propagons are diluted to one unit/cell, and upon plating in the absence of GdnHCl, all cells that form white colonies must have inherited a propagon. Thus, the number of white colonies corresponds to the number of

propagons in the original cell. Importantly, the number of propagons in each strain was similar prior to GdnHCl treatment, and GdnHCl treatment similarly reduced propagon number in all strains (Figure 2A-C, 0 time point, Figure 4E, columns 2-4). Strains containing a repeat deletion (R1-5) return to equilibrium slightly slower than wildtype, albeit this difference is not significant (Figure 2A). Strains containing a repeat expansion, however, recover significantly faster than wildtype (Figure 2B), suggesting that the number of repeats is positively correlated with the ability of prion aggregates to amplify. In addition, strains containing the RPR deletion (Δ RPR), recover significantly slower than wildtype (Figure 2C), indicating that, like the repeats, the RPR also affects the rate of prion amplification.

The ability of the prion state to return to equilibrium following near curing *in vivo* is a function of both conversion, the rate at which soluble Sup35 adds onto existing templates, and fragmentation, the rate at which Hsp104 disassembles existing aggregates (Tanaka et al., 2006). To determine which of these steps was altered in the repeat variant strains, we first directly monitored conversion using a fluorescent read-through reporter assay (DiSalvo et al., 2011; Satpute-Krishnan and Serio, 2005). In this assay, [*psi*⁻] repeat variant strains containing the reporter GST(UGA)YFPNLS were mated to wildtype [*psi*⁻] or [*PSI*⁺] strains. While the reporter is not expressed in the repeat variant strain due to the absence of [*PSI*⁺], the soluble Sup35 protein in this strain join existing Sup35 aggregates in the [*PSI*⁺] mating partner when the cells fuse. The efficiency with which soluble, repeat variant Sup35 joins existing aggregates can then be

inferred by nuclear fluorescence intensity in the zygote because the aggregation of Sup35 is associated with a translation termination defect (Figure 3A). As expected, when a wildtype [*psi*⁻] strain expressing the read-through reporter was crossed to a [*PSI*⁺] strain, there was about a four-fold increase in fluorescence intensity compared to the cross to a [*psi*⁻] strain (Figure 3B, compare lanes 1 and 5), validating the assay. The repeat deletion, RPR deletion, and repeat expansion all showed similar levels of fluorescence when crossed to a [*psi*⁻] strain (Figure 3B, lanes 1-4), suggesting that Sup35 in these crosses remains soluble. When crossed to a [*PSI*⁺] strain, both the repeat deletion and the RPR deletion showed similar levels of fluorescence compared to wildtype (Figure 3B, lanes 5-7), indicating that Sup35 monomers containing a repeat or RPR deletion are not impaired in their ability to add onto existing aggregates. However, the repeat expansion showed a significant increase in nuclear fluorescence intensity, compared to wild type (Figure 3B, compare lanes 5 and 8), indicating that the repeat expansion may be more efficient at joining existing aggregates. Because differences in fragmentation by Hsp104 affect the accumulation of templates and thereby affect the conversion efficiency, we wanted to determine if the difference in nuclear fluorescence intensity observed with the repeat expansion was directly related to its ability to join aggregates or if it was a downstream effect of a change in fragmentation. Towards this end, we repeated the wildtype and repeat expansion crosses to a [*PSI*⁺] strain in the presence of GdnHCl, which inhibits Hsp104 activity and therefore prevents changes in aggregate number, which should eliminate any bias of fragmentation in this assay. Under these conditions,

there was no difference in nuclear fluorescence intensity in comparison with a cross between wildtype [*psi*⁻] and [*PSI*⁺] strains (Figure 3C), indicating that the repeats do not affect conversion directly but likely act through an Hsp104-dependent event, suggesting that the repeats may affect fragmentation efficiency.

Changes in the rates of conversion and fragmentation lead to predictable changes in steady-state aggregate size, aggregate number, and soluble Sup35. To further distinguish whether the repeats and the RPR region affect conversion or fragmentation, we next assessed Sup35 aggregate size in strains propagating [*PSI*⁺] through these variants. Strains containing four, five or the wildtype number of repeats have similar median aggregate size, but strains containing a repeat expansion showed a decrease in overall aggregate size, with the effect being more severe in R2E2 than R2E1 (Figure 4A). Smaller aggregates could be attribute to decreased conversion or increased fragmentation (DiSalvo et al., 2011; Holmes et al., 2014). If the expansion decreased conversion, we would expect an increase in soluble Sup35. However, if the repeat expansion increased fragmentation, we would expect an increase in the number of propagons, resulting in more templates for monomer addition, and thereby less soluble Sup35. Indeed, compared to wildtype, the repeat expansions result in an increase in propagon number (Figure 4E compare WT and R2E2), and a decrease in soluble Sup35 (Figure 4D compare WT and R2E1).

We next asked if the effects seen in the repeat expansion were due to an increase in total repeat number, or solely due to the presence of extra copies of

the second repeat. To do this, we constructed a strain (R2E2 Δ 4-5), which contains the repeat expansion, but has the wildtype number of repeats (Figure 1A). Compared to wildtype, R2E2 Δ 4-5 showed a loss of larger aggregates (Figure 4C). Consistent with the functional engagement of small but not large Sup35 aggregates in the termination reaction (Pezza et al., 2014), this strain appeared pinker on rich medium and had poor growth on media lacking adenine (Figure 1C). Together, these observations indicate that the effect of repeat expansion in the wildtype context documented above cannot be attributed solely to the presence of an additional copy of the second repeat and that repeat number alone is not sufficient to fully support [*PSI*⁺] propagation. Rather, both total repeat number and precise repeat sequence play a role in prion aggregate dynamics.

In contrast to the effects observed in strains containing an increased number of repeats, deletion of the RPR had no effect on the accumulation of propagons (Figure 4E) but did increase the accumulation of soluble Sup35 relative to the wildtype strain (Figure 4D), which could indicate either a defect in conversion and/or in fragmentation. To distinguish between these possibilities, we monitored Sup35 aggregate size in these strains. In a strain disrupted for the RPR, Sup35 aggregate size increased in comparison with wildtype, consistent with a defect in fragmentation (Figure 4B). Intriguingly, while there were no significant changes in propagon number or in soluble Sup35 in an R1-5 repeat deletion compared to wildtype (Figure 4C and 4D, compare WT to R1-5), reducing the number of repeats in the Δ RPR context (R1-5 Δ RPR) led to a similar

increase in Sup35 aggregate size (Figure 4B), an increase in the accumulation of soluble Sup35, and a reduction in the accumulation of propagons relative to either the wildtype strain or to each single deletion (Figure 4D, E), consistent with a further decrease in fragmentation efficiency. Surprisingly, the further deletion of another repeat in R1-4, which would be expected to more severely impair fragmentation, broadens rather than increases the range of Sup35 aggregate sizes (Figure 4B), an effect that likely results from the change in chaperone expression that is associated with R1-4 toxicity (discussed in Chapter 3). Nevertheless, the additional deletion of the RPR in the R1-4 context exacerbates the impact of repeat deletion on $[PSI^+]$ propagation, pushing the prion propagation defect in the R1-5 Δ RPR strain to complete curing in the R1-4 Δ RPR strain (Figures 1B, 4B). Thus, deletion of the RPR, along with the positive correlation between repeat expansions and fragmentation efficiency (Figure 4) and the ability of overexpressed Hsp104 to suppress the prion propagation defect of a repeat deletion (Borchsenius et al., 2001), reinforces a role for the oligopeptide repeats in promoting aggregate fragmentation.

The repeat region alters the efficiency of the interactions between Sup35 aggregates and molecular chaperones

We next sought to determine the precise mechanism by which these sequence elements are acting to promote the fragmentation of Sup35 prion aggregates. Because the repeat expansion constructs were difficult to express at wildtype levels, we first considered the possibility that these constructs might

induce a stress response, resulting in increased chaperone levels in strains carrying the repeat expansion constructs. To determine if this was the case, we examined the expression levels of Hsp104 and its co-chaperones Ssa1 (Hsp70) and Sis1 (Hsp40) by quantitative immunoblot. The R1-5, R2E1 and Δ RPR strains had similar levels of Hsp104, Ssa1, and Sis1 to the wildtype strain (Figure S2). Therefore, elevation of the chaperones mediating aggregate fragmentation cannot explain the changes in fragmentation efficiency in these strains.

We next considered that the repeats and RPR may act on fragmentation by altering the physical properties of prion aggregates, such as their thermodynamic stability, as has been observed with the $[PSI^+]$ -No-More mutant, G58D (DiSalvo et al., 2011; Verges et al., 2011). To determine the relative thermodynamic stability of prion aggregates in repeat variant strains, we incubated cell lysates at increasing temperature in the presence of 2% SDS and determined the amount of Sup35 protein released from aggregates at each temperature. We saw no difference in the amount of Sup35 protein that was released from aggregates at 65°C, 72.5°C, or 80°C in the repeat deletion (R1-5), repeat expansion (R2E1), or RPR deletion compared to wildtype (Figure 5), indicating that the repeat and RPR variants do not affect the thermodynamic stability of Sup35 aggregates. In addition, the similar thermodynamic stability suggests that changes in the composition of these sequence elements do not result in the appearance of different prion strains *in vivo*.

Because the relative levels of chaperones and thermodynamic stability of Sup35 aggregates are not altered in the repeat or RPR variant strains, we next

considered the possibility that the repeat region may affect the efficiency by which aggregates are acted on by the molecular chaperone, Hsp104. Hsp104 binds to prion aggregates and threads a monomer through its central pore, leading to the destabilization and subsequent fragmentation of the aggregate (Haslberger et al., 2008; Lum et al., 2004). We hypothesized that the repeat region may affect the ability of Hsp104 to recognize and bind to prion aggregates and/or its ability to process substrates through its threading mechanism.

Because the conversion and fragmentation steps of prion propagation are interconnected, we developed a heterologous system lacking the conversion step to separately assess the contribution of these sequence elements to Hsp104-mediated disassembly. We created a series of reporter constructs consisting of firefly luciferase fused to *Renilla* luciferase-GFP. Firefly luciferase is a well-characterized model substrate, which denatures and aggregates upon heat shock and is disaggregated and reactivated by Hsp104 (Parsell et al., 1994), and *Renilla* luciferase behaves similarly (Figure S3A). In addition the GFP tag on this construct allows us to use a dual assay to monitor Hsp104 activity: a biochemical assay that reports on the folded state of luciferase and a visual assay that reports on the aggregation state of the protein (Abrams and Morano, 2013). At the boundary between the two luciferase proteins, we inserted variants of the N domain of Sup35: wildtype, R1-4, R2E2, and Δ RPR, opting for the more severe deletions and expansions to promote assay sensitivity. The toxicity of these variants in the native Sup35 context will not impact this assay because we conducted these experiments in [*psi*⁺] cells and because fusing a protein to the N-

terminus of Sup35, as occurs in this reporter protein, blocks its ability to adopt the prion state (Satpute-Krishnan and Serio, 2005).

We first wanted to test whether recovery of our reporter construct following heat shock was dependent on Hsp104. We pretreated cultures containing either wildtype Hsp104 or an Hsp104 deletion at 37°C to allow increased expression of chaperones, followed by heat shock at 40°C to denature luciferase. Cells were allowed to recover at 30°C in the presence of cycloheximide to monitor the fate of existing luciferase reporter. Immediately following heat shock, both firefly and *Renilla* luciferase activity as measured by luminescence following addition of their substrates dropped to ~30% of the pre-heat shock level (Figure S3B), consistent with previous reports (Abrams and Morano, 2013; Parsell et al., 1994), and indicating that our reporter construct is efficiently heat denatured. During recovery at 30°C, both firefly and *Renilla* luciferase activity recovered with similar kinetics in cells containing wildtype Hsp104, reaching at least 80% activity within one hour (Figure S3B). As expected, in Δ Hsp104 cells, neither firefly nor *Renilla* luciferase activity recovered, indicating that recovery of both proteins in the context of this reporter is dependent on Hsp104 (Figure S3B). Next, we monitored the aggregation state of the reporter by microscopy. Prior to heat shock, GFP fluorescence was diffuse throughout the cell, consistent most of the proteins being soluble (Figure S3F). Immediately following heat shock, all cells contained GFP foci, consistent with denaturation and aggregation of the protein. These foci quickly recovered in cells containing wildtype Hsp104, with most foci being resolved within one hour (Figure S3F, right, Figure 6A). In Δ Hsp104 cells,

the GFP foci persisted, with all cells still containing foci at ninety minutes (Figure S3F, left), indicating that similar to the activity assay, disaggregation of luciferase following heat shock is dependent on Hsp104.

We next wanted to compare the recovery rates of luciferase reporter constructs containing different numbers of repeats within the Sup35N fragment. Importantly, the luciferase recovery by both the activity and fluorescent assays in all strains was dependent on Hsp104, regardless of which repeat variant the reporter construct contained (Figure S3C-F). Using these strains, we first compared the relative rates of luciferase disaggregation. For this experiment, we monitored the fluorescence pattern over a time course after heat shock by microfluidics in order to increase temporal resolution. For the reporter containing Sup35N with the wildtype number of repeats, half the cells resolved their foci in ~70 minutes, with all foci resolved by 102 minutes (Figure 6A). Foci containing the reporter protein containing the repeat deletion (R1-4), which is predicted to reduce the rate of fragmentation of Sup35 aggregates based on the loss of $[PSI^+]$ in the presence of an RPR deletion (Figure 1B), were resolved more slowly than those containing the wildtype Sup35N region, with half the cells recovering their foci in ~87 minutes and full resolution not occurring until 138 minutes (Figure 6A). In contrast, the foci containing the reporter protein with a repeat expansion (R2E2) were resolved significantly faster compared to those containing the wildtype Sup35N region, with half the cells resolving their foci by ~62 minutes and full resolution occurring at 84 minutes (Figure 6A), again consistent with the higher rates of fragmentation of Sup35 aggregation in $[PSI^+]$ version of these

strains. In addition, the faster rate of luciferase disaggregation observed with the repeat expansion is consistent with increased rate of prion amplification as determined by propagon recovery (Figure 2B).

We next determined the relative rates of luciferase refolding in the repeat expansions and deletions. Importantly, both the levels of reporter protein and activity in the absence of heat shock were identical for all of the variants studied, indicating similar efficiencies of protein maturation and stability (Figure S5A, B). In contrast to the relative rates of disaggregation, the reporter containing the repeat deletion refolded significantly faster than wildtype (Figure 6B, S4A), and the repeat expansion refolded significantly slower than wildtype (Figure 6C, S4B), suggesting that the rate of refolding is inversely related to repeat number. Because both disaggregation and refolding require binding to Hsp104 (Figure S3), the uncoupling of these two rates suggests that the repeats act at a step downstream of binding that more directly impacts substrate refolding than disaggregation, such as substrate threading. In this scenario, refolding would require complete unfolding of each monomer by translocation through the central pore of Hsp104, but the extraction of a few monomers from an aggregate could destabilize the complex, leading to complete disaggregation without refolding.

We next determined if the RPR was exerting a similar effect on chaperone activity. In contrast to the repeat deletion, there was not a significant difference in disaggregation or refolding of the reporter construct containing Sup35N Δ RPR compared to wildtype (Figure 6A 6D, S4C). This difference in recovery kinetics compared to the repeat deletions suggests that the RPR deletion is affecting

fragmentation through a mechanism that is distinct from that of the repeats. One possible explanation for how the RPR is affecting fragmentation could be through one of Hsp104's co-chaperones: Ssa1 (Hsp70) or Sis1 (Hsp40). Indeed, the RPR contains a predicted binding site for Ssa1 (GNYKNFN) (Van Durme et al., 2009). In this model, deletion of the RPR would prevent or decrease Ssa1 binding to prion aggregates, thereby decreasing the efficiency through which aggregates recruit Hsp104 (Winkler et al., 2012). This model accounts for the lack of differences between wildtype and Δ RPR luciferase reporter constructs, as in this context Hsp104 (and consequently) Ssa1 are likely recruited to the luciferase region(s) and not to Sup35N.

Discussion

Our data support the idea that the oligopeptide repeats and the RPR are important for fragmentation of prion aggregates. These regions do not affect the physical properties of the aggregates themselves, but rather their interactions with cellular chaperones. For the repeat region, we propose that these sequence elements affect the ability of Hsp104 to thread Sup35 through its central pore. Hsp104's central pore contains flexible aromatic residues, whose primary role is to interact with the substrate. Conformational changes in these residues then provide the power stroke, allowing Hsp104 to pull its substrate through the central pore (Tyedmers et al., 2010). We propose that the low sequence complexity of the repeat region provides fewer architectural elements for Hsp104

to interact with, resulting in stalling of the enzyme when it reaches the repeats. Repeated engagement of Hsp104 at these regions then provides a force which destabilizes prion aggregates, leading to their fragmentation. In this model, Hsp104 is easily able to process Sup35 monomers with fewer repeats, leading to their extraction with fewer power strokes and resulting in less aggregate destabilization. As a consequence, more monomers must be directly extracted by Hsp104 to resolve the aggregates, a requirement that promotes the faster refolding of the protein. In contrast, when repeat expansions are present, Hsp104 must engage more frequently with the substrate, and this repeated force causes an increased destabilization of Sup35 aggregates. As a consequence, monomers may be released from aggregates without translocation through the central pore of Hsp104, prolonging their persistence in a misfolded state. Consistent with this model, the force required to unfold prion fibers in single-molecule optical trapping experiments increases with repeat number, and R2E2 Sup35 fibers are able to refold following mechanical unfolding, whereas repeat deletion (R Δ 2-5) fibers cannot quickly refold, leading to fiber stability in the former case and fiber breakage in the latter, (Dong et al., 2010). These observations likely correlate with the power stroke of Hsp104, which is transient and repetitive. Indeed, the ability of substrates to be processed by AAA+ ATPases is essentially a competition between the unfolding of the substrate by the enzyme and its ability to refold in between each power stroke (Martin et al., 2005). Thus, the ability of the repeat expansion to quickly refold supports our idea that this mutant may act to stall the chaperone machinery.

Similar stop-transfer sequences have been identified in the context of other AAA+ ATPase machines: ClpXP and the proteasome. For example, the transcription factors NF- κ B in higher eukaryotes and cubitus interruptus (Ci) in *Drosophila* both exist as a full length inactive precursor in the cytosol, which is partially degraded by the proteasome to produce an active truncated product, which then translocates to the nucleus and activates target genes (Aza-Blanc et al., 1997; Orian et al., 1999). The partial degradation of these proteins is dependent on two features: a stretch of simple sequence proximal to a tightly folded domain. When the proteasome reaches the simple sequence, the lack of architectural features reduces the amount of force the proteasome can exert to thread the substrate. This reduced force, combined with the nearby tightly folded domain, results in stalling of the proteasome and the release of a partially degraded product (Lee et al., 2001; Tian et al., 2005). This mechanism has been reported across diverse species, explaining the activation of the yeast transcription factors Spt23 and Mga2 as well as the degradation recalcitrant EBNA1 protein of the Epstein-Barr virus (Hoyt et al., 2006; Rape and Jentsch, 2002).

In the case of Sup35, we propose that processing of Sup35 by Hsp104 also occurs through a similar mechanism with the oligopeptide repeat region acting as the simple sequence and the Q/N rich region, which is the site of intermolecular contacts in the amyloid (Toyama et al., 2007), acting as the tightly folded domain. Intriguingly, [PSI⁺] is extremely sensitive to mutations in the Gly-Gly pair of repeat 2 (G58X, G59X), but it not as sensitive to mutations in the Gly-

Gly pair of repeats 1, 4 or 5 (repeat 3 does not contain a Gly-Gly pair) (DiSalvo et al., 2011; Doel et al., 1994; Marchante et al., 2013). One explanation for this finding is that the Gly-Gly pair in repeat 2 is uniquely positioned in relationship to the amyloid core (~22 amino acids separate G58/59 and the amyloid core) such that G58/59 reaches the aromatic paddles of Hsp104 at the same time that the amyloid core reaches the pore opening, a distance predicted to accommodate 14-24 amino acids (Hoyt et al., 2006; Kenniston et al., 2005; Lee et al., 2010; Toyama et al., 2007). Thus, the increase in architecture created by mutating these residues may result in features for Hsp104 to more efficiently interact with at the same time that more energy is required to unfold the aggregation domain, reducing stalling, allowing for more efficient enzyme processing, and resulting in $[PSI^+]$ destabilization in a way that is mechanistically similar to a repeat deletion.

Previous work has suggested that the primary sequence composition, and not the repeated sequence *per se*, was the important factor in supporting prion propagation. In these studies, mutants were generated by scrambling the sequence of the repeat domain but by leaving the amino acid composition intact. Many, but not all, of these mutants were able to support $[PSI^+]$. However, high levels of $[PSI^+]$ loss were observed, and the mutants could not template wildtype Sup35 (Ross et al., 2005; Toombs et al., 2011), suggesting that the primary sequence composition of this region is necessary but not sufficient for efficient prion propagation. Indeed, the spacing of Q/N residues with aromatic or hydrophobic residues is an important determinant for fiber stability and fragmentability (Alberti et al., 2009; Alexandrov et al., 2012, 2008). In agreement

with this model, our studies with the R2E2Δ4-5 mutant suggest that both sequence composition and specific repeat structure are important for efficient propagation.

Interestingly, the precise number of repeats is conserved across a variety of amyloids. In all yeast species in which the Sup35 homologue has been shown to be capable of forming a prion in the *S. cerevisiae* cytoplasm, the Sup35 protein contains between five and six repeats (Nakayashiki et al., 2001; Parham et al., 2001; Santoso et al., 2000). Similarly, the mammalian prion protein (PrP) contains five copies of an octarepeat (van Rheede et al., 2003). In addition, bacterial functional amyloids show similar requirements for repeated elements, with CsgA containing 5 copies of a hexapeptide repeat, and FapC containing three copies of a repeat (Cherny et al., 2005; Dueholm et al., 2010). One possible explanation for this conservation of repeats across diverse species is that the precise number of repeats has been evolutionarily optimized to allow for efficient maintenance of both the amyloid and non-amyloid states. Our results suggest that the oligopeptide repeat region is crucial for proper aggregate amplification, and the amplification efficiency can be directly correlated with an ability to maintain the prion state. When too few repeats are present, aggregates cannot be amplified efficiently, leading to an inability to maintain the amyloid state. However, when too many repeats are present, as in the case of repeat expansions, amplification is increased, leading to a reduction in the ability of cells to maintain the non-amyloid state. Indeed, the ability to maintain both the amyloid and non-amyloid states is critically important considering the idea that

the prion form may confer either an advantage or disadvantage depending on growth conditions (True and Lindquist, 2000). In this case, the ability to switch between [PRION+] and [prion-], or *vice versa* and subsequently maintain either state can be thought of as a bet-hedging mechanism to ensure species survival in a variety of environmental conditions (Halfmann et al., 2010). In support of this idea, yeast prions are enriched for proteins involved in transcription and translation, suggesting that switching between prion and non-prion states may provide an epigenetic mechanism for cells to switch quickly between transcriptional or translational programs (Alberti et al., 2009; Halfmann et al., 2010). Indeed, this view point is supported by data that the [MOT3] yeast prion can be advantageous to cells in certain conditions by promoting a multicellular phenotype (Holmes et al., 2013).

Similar to our results with Sup35, repeat deletions and expansions in mammalian PrP and polyQ expansions in Huntington's Disease result in specific disease phenotypes. For example, individuals who express PrP with a repeat expansion or huntingtin with polyQ expansions have an earlier age of disease onset and faster disease progression (Guo et al., 2008; Jansen et al., 2011; Mauro et al., 2008; Williams and Paulson, 2008). One possible explanation for this increased rate of disease progression is that as for Sup35 repeats, PrP repeat expansion results in increased aggregate amplification, resulting in aggregate accumulation and subsequent toxicity at an earlier age. While a AAA+ ATPase responsible for fragmentation in mammals has not been identified, mathematical *ex vivo* models suggest that there must exist a similar mechanism

for fragmentation (Krauss and Vorberg, 2013; Masel et al., 2005). In addition, polyQ sequences have been demonstrated to decrease the processivity of the proteasome in a length dependent manner (Kraut et al., 2012; Venkatraman et al., 2004; Verhoef et al., 2002), suggesting that the interaction of repeat sequences with cellular AAA+ ATPases may represent a conserved mechanism across a variety of species that promotes amyloid stability.

Table 1: Plasmids

Name	Description
SLL6686	pRS306-P _{SUP35} Sup35
SB803	pRS306-P _{SUP35} Sup35R1-2
SB804	pRS306-P _{SUP35} Sup35R1-3
SB775	pRS306-P _{SUP35} Sup35R1-4
SB776	pRS306-P _{SUP35} Sup35R1-5
SB787	pRS306-P _{SUP35} Sup35R2E1
SB883	pRS303-P _{SUP35} Sup35R2E1
SB859	pRS306-P _{SUP35} Sup35R2E2
SB884	pRS303-P _{SUP35} Sup35R2E2
SB549	pRS306-P _{SUP35} Sup35R1-4ΔRPR
SB550	pRS306-P _{SUP35} Sup35R1-5ΔRPR
SB777	pRS306-P _{SUP35} Sup35ΔRPR
SB1008	pRS306-P _{SUP35} Sup35R2E2Δ4-5
SB910	pRS304-P _{GPD} GST(UGA)YFPNLS
SB994	pRS306-P _{GPD} Firefly-Sup35N-Renilla-GFPGAR
SB975	pRS306-P _{GPD} Firefly-Sup35NΔRPR-Renilla-GFPGAR
SB976	pRS306-P _{GPD} Firefly-Sup35NR2E2-Renilla-GFPGAR
SB985	pRS306-P _{GPD} Firefly-Sup35NR1-4-Renilla-GFPGAR
SB986	pRS306-P _{GPD} Renilla-GFPGAR

Table 2: Primers

Name	Sequence
5BamHISup35	5'- GGATCCATGTCGGATTCAAACCAAGGC-3'
3R1-2EcoRV	5'-GATATCTTGCAAATTGTTATTGTAGTTGAAGTTTTTGTA ATTTCCACGATTGTACTGTT-3'
3R1-3EcoRV	5'- GATATCTTGCAAATTGTTATTGTAGTTGAAGTTTTTGTA ATTTCCACGATTATACTGTT-3'
3R1-4EcoRV	5'- GATATCTTGCAAATTGTTATTGTAGTTGAAGTTTTTGTA ATTTCCACGATTGTACTGTT-3'
3R1-5EcoRV	5'-GATATCTTGCAAATTGTTATTGTAGTTGAAGTTTTTGTA ATTTCCACGATTGAATTGCT-3'
3Sup35R1-6EcoRV	5'- GATATCCGCCACCTTGTGGATTGAATTC-3'
R2E1 insertF	5'- GTGGCTATCAACAGTACAACCAACAAG-3'
R2E1 insertR	5'- TTGGTTGTAAGTTGATAGCCACCTTG-3'
R2BstXI QCF	5'-CAAAATTACCAAGGTTATTCTGGGTATCAACAAGGTGG CTATCAACAGTAC-3'
R2BstXI QCR	5'- GTAAGTTGATAGCCACCTTGTGATACCCAGAATAAC CTTGGTAATTTG-3'
5EcoRI Citrine	5'-GATATCATGTCTAAAGGTGAAGAATTATTC-3'
3ClaI CitrineNLS	5'-ATCGATTTATCCCTTTGGGTCTTCAACCTTTCTCTTCT TCTTTGGTGGGGTAGAGTGCCCTTTGTACAATTCATCCATAC CATG-3'
5XbaI firefly	5'-TCTAGAATGGAAGATGCCAAAACATTAAG-3'
3BamHI Firefly	5'-GGATCCACCTTGAGACTGTGGTTGGAAAC-3'
5BamHIGs3 Sup35N	5'-GGATCCGGTAGTGGTAGTGGTAGTATGTCGGATTCAAA CCAAGGC-3'
3BamHI Sup35N	5'-GGATCCACCTTGAGACTGTGGTTGG-3'
5BamHIGS3 Renilla	5'-GGATCCGGTAGTGGTAGTGGTAGTATGACTTCGAAAGTT TATGATCC-3'
3EcoRI Renilla	5'-GAATTCCTTGTTCAATTTTGAGAACTC-3'
5EcoRI GS3 GFP	5'-GAATTCGGTAGTGGTAGTGGTAGTATGGCTAGCAAAGG AGAA-3'
3XhoI GArGF P	5'-CTCGAGTTAACCTGCACCTGCACCACCACCTGCACCTGC TTTGTATAGTTCATCCATGCC-3'
5XbaIRenilla	5'-TCTAGAATGACTTCGAAAGTTTATGATCC-3'
5Sup35Nrep ck	5'-TGTCGGATTCAAACCAAGGCAACAATCAGCAAAAC-3'

3Sup35Nrep ck	5'-GCCAACCTTCTTGGTAGCATTGGCCAACTTGATACC-3'
F4-Psup35	5'-CTTCTCTTGAAAGACTCCATTGTACTGTAACAAAAAGCGG GAATTCGAGCTCGTTTAAAC-3'
R2-PMFA1	5'-GCTGGTAGTTTTGCTGATTGTTGCCTTGGTTTGAATCCGA CATGGATCCTTCTATTGAT-3'

Table 3: Strains

Strain	Genotype	Plasmids Integrated	Reference
SLL2606	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112</i>	-	Chernoff et al. 1995
SLL2119	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112</i>	-	Chernoff et al. 1995
SY2072	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-2</i>	SB803	This study
SY2073	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-3</i>	SB804	This study
SY2057	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-4</i>	SB775	This study
SY2022	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-5</i>	SB776	This study
SY2247	<i>MATa [PSI⁺] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E1 trp1-289 ura3-52 leu2-3, 112 SUP35R2E1</i>	SB787, SB883	This study
SY2300	<i>MATa [PSI⁺] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E2 trp1-289 ura3-52 leu2-3, 112 sup35::P_{MFA1}SUP35R2E2::KANMX6</i>	SB859, SB884	This study
SY1629	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-4ΔRPR</i>	SB549	This study
SY1633	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-5ΔRPR</i>	SB550	This study
SY2023	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35ΔRPR</i>	SB777	This study
SY2808	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R2E2Δ4-5</i>	SB1008	This study
SY2393	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289::TRP1::P_{GPD}GST(UGA)YFPNLS ura3-52 leu2-3, 112</i>	SB910	This study
SY2461	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289::TRP1::P_{GPD}GST(UGA)YFPNLS ura3-52 leu2-3, 112 SUP35R1-5</i>	SB776, SB910	This study
SY2463	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289::TRP1::P_{GPD}GST(UGA)YFPNLS ura3-52 leu2-3, 112 SUP35ΔRPR</i>	SB777, SB910	This study
SY2465	<i>MATa [psi⁻] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E1 trp1-289::TRP::P_{GPD}GST(UGA)YFPNLS ura3-</i>	SB787, SB910	This study

	52 <i>leu2-3</i> , 112 <i>SUP35R2E1</i>		
SY2603	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35N-Renilla-GFP leu2-3, 112</i>	SB994	This study
SY2637	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35NΔRPR-Renilla-GFP leu2-3, 112</i>	SB975	This study
SY2640	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35NR2E2-Renilla-GFP leu2-3, 112</i>	SB976	This study
SY2666	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35NR1-4-Renilla-GFP leu2-3, 112</i>	SB985	This study
SY2625	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35N-Renilla-GFP leu2-3, 112 hsp104::LEU2</i>	SB994	This study
SY2651	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35NΔRPR-Renilla-GFP leu2-3, 112 hsp104::LEU2</i>	SB975	This study
SY2654	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35NR2E2-Renilla-GFP leu2-3, 112 hsp104::LEU2</i>	SB976	This study
SY2679	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Firefly-SUP35NR1-4-Renilla-GFP leu2-3, 112 hsp104::LEU2</i>	SB985	This study
SY2694	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Renilla-GFP leu2-3, 112</i>	SB986	This study
SY2703	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{GPD}Renilla-GFP leu2-3, 112 hsp104::LEU2</i>	SB986	This study
SY2213	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-4</i>	SB775	This study
SY2215	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-5</i>	SB776	This study
SY2212	<i>MATa [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35 ΔRPR</i>	SB777	This study
SY2302	<i>MATa [psi⁻] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E1 trp1-289 ura3-52 leu2-3, 112 SUP35R2E1</i>	SB787, SB883	This study

SY2305		SB859, SB884	This study
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Materials and Methods

Plasmids

All plasmids used in this study are listed in Table 1. All primer sequences are listed in Table 2. All constructs were confirmed by DNA sequencing.

Repeat deletion plasmids

Repeat deletions R1-2 (SB803), R1-3 (SB804), R1-4 (SB775), R1-5 (SB776), and Δ RPR (SB777) were constructed by amplification of a *Bam*HI/*Eco*RV Sup35 fragment with primers 5BamHISup35 and 3R1-2EcoRV, 3R1-3EcoRV, 3R1-4EcoRV, 3R1-5EcoRV, and 3Sup35R1-6EcoRV respectively and using SLL6686 (pRS305-P_{SUP35}Sup35) as a template. *Bam*HI/*Eco*RV fragments were inserted into SLL6686. R1-4 Δ RPR (SB549) and R1-5 Δ RPR (SB550) were constructed as previously described (Parham et al., 2001).

Repeat expansion plasmids

R2E1 (SB787) was constructed by annealing oligos R2E1 insertF and R2E1 insertR, and insertion of the resulting product into the *Bst*XI site of SLL6686. R2E2 (SB859) was constructed by QuikChange (Stratagene) of SB787 using primers R2 *Bst*XI QCF and R2 *Bst*XI QCR, followed by insertion of annealed oligos R2E1 insertF and R2E1 insertR into the resulting *Bst*XI site. SB883 and

SB884 were constructed by insertion of the R2E1 and R2E2 ORFs, respectively, as an *EcoRI/SacI* fragment into pRS303. R2E2 Δ 4-5 (SB1008) was synthesized as a *BamHI/BseRI* fragment (Genewiz) and inserted into SLL6686.

Conversion read-through plasmid

YFPNLS ORF was generated as a PCR fragment using the primers 5EcoRI Citrine and 3Clal CitrineNLS and inserted into SB531 (pRS304-P_{GPD}GST(UGA)DsRedNLS, (Pezza et al., 2009)) using *EcoRI* and *Clal* sites to generate pRS304-P_{GPD}GST(UGA)YFPNLS.

Dual Luciferase constructs

SB994, SB975, SB976, and SB985 contain the GPD promoter driving firefly luciferase-Sup35N-Renilla Luciferase- GFPGAR. A three repeat glycine serine linker separates each of the ORFs. ORFs for Firefly luciferase (*XbaI/BamHI* PCR fragment generated using 5XbaI Firefly and 3BamHI Firefly), Sup35N (*BamHI/BamHI* PCR fragment generated using 5BamHIGS3 Sup35N and 3BamHI Sup35N), *Renilla* luciferase (*BamHI/EcoRI* PCR fragment generated using 5BamHIGS3 Renilla and 3 EcoRI Renilla), and GFPGAR (*EcoRI/XhoI* PCR fragment generated using 5EcoRIGS3 GFP and 3XhoIGAR GFP), were inserted into SB237 (pRS305-P_{GPD}). For Sup35N Rwt, R1-4, R2E2, and Δ RPR, PCR templates were SLL6686, SB775, SB859, and SB777 respectively.

SB986 (pRS306-P_{GPD}Renilla-GFP_{GA}r) contains *Renilla* Luciferase-GFP_{GA}r, with the open reading frames separated by a three repeat glycine-serine linker. *Renilla* luciferase (*Xba*I/*Eco*RI PCR fragment generated using 5*Xba*I Renilla and 3*Eco*RI Renilla), and GFP-Gar (*Eco*RI/*Xho*I PCR fragment generated using 5*Eco*RI_{GS3} GFP and 3*Xho*I_{GA}r GFP) were inserted into SB237 (pRS305-P_{GPD}).

Strain Construction

All strains used in this study are listed in Table 3 and are derivatives of 74-D694. All [*PSI*⁺] strains are propagating [*PSI*⁺]^{strong}, unless otherwise indicated.

Repeat Variant Strains

SY2072 (R1-2), SY2073 (R1-3), SY2057 (R1-4), SY 2022 (R1-5), SY1629 (R1-4ΔRPR), SY1633 (R1-5ΔRPR), SY2023 (ΔRPR), and SY2808 (R2E2Δ4-5) were constructed by two-step replacement at the *SUP35* locus following integration of *Mlu*I-digested SB803, SB804, SB775, SB776, SB549, SB550, SB777, and SB1008 respectively, selection on SD-Ura, followed by counter selection on 5-FOA. Strains were screened for repeat number by PCR using 5Sup35Nrepck and 3Sup35repck, confirmed by sequencing, and selected to express Sup35 at endogenous levels by quantitative immunoblot. SY2247 (R2E1) was constructed by two-step replacement at the Sup35 locus following integration of *Mlu*I-digested SB787, selection on SD-Ura and counter-selection on 5-FOA. Because Sup35

expression was reduced relative to a wildtype strain, a second copy of R2E1 was integrated at the *HIS3* locus by transforming an *AfeI*-digested SB883 and selecting on SD-His. Strains were screened for repeat number by PCR using 5Sup35Nrepck and 3Sup35repck, confirmed by sequencing, and selected for endogenous levels of Sup35 expression. SY2300 (R2E2) was constructed by two-step replacement at the *SUP35* locus following integration of *MluI*-digested SB859, selection on SD-Ura, and counter selection on 5-FOA. Because Sup35 expression was reduced relative to a wildtype strain, a second copy of R2E2 was integrated at the *HIS3* locus by transforming an *AfeI*-digested SB883 and selecting on SD-His. In order to obtain endogenous levels of Sup35 expression, the promoter at the endogenous locus was also replaced with P_{MFA1} by integration of a PCR-generated cassette, using F4-PSup35 and R2-PMFAI primers, with SB526 (pFA6a-KanMX6- P_{MFA1} , (Pezza et al., 2009)) as a template and selection on YPD+G418. Strains were screened for repeat number by PCR using 5Sup35Nrepck and 3Sup35repck, confirmed by sequencing, and selected for endogenous levels of Sup35 expression.

[*psi*⁻] versions of repeat variant strains were generated by curing on YPD + 3mM Guanidine HCL (GdnHCl) and selection for colony color (Eaglestone et al., 2000).

SY2335, SY2336, SY2468, and SY2337 were constructed by integration of *PpuMI*-digested SLL6682 into SLL2606, SY2057, SY2247 and SY2300 respectively, selection on SD-Ura, and screening by colony color. [*PSI*⁺] was confirmed by SDD-AGE.

Conversion strains

SY2393 was constructed by integrating a *Ppu*MI-digested SB910 (pRS304- P_{GPD} GST(UGA)YFPNLS) in SLL3250, selecting on SD-Trp, and screening by fluorescence, followed by prion curing by 3mM guanidine HCl (GdnHCl). SY2461, SY2463, and SY2465 were constructed by mating SY2393 to SY2215, SY2212, and SY2302 respectively, followed by sporulation, dissection and selection on SD-Trp. Repeat number in tetrads was screened by PCR using primers 5Sup35Nrepck and 3Sup35Nrepck.

Luciferase strains

SY2603, SY2637, SY2640, SY2666, and SY2694 were constructed by integration of *Stu*I-digested SB994, SB975, SB976, SB985, and SB986 respectively. Strains were screened by luminescence and fluorescence. Hsp104 disruptions SY2625, SY2651, SY2654, SY2679, and SY2703 were constructed by integration of a *Pvu*II/*Bam*HI-digested fragment of pYABL5 (S. Lindquist) into SY2603, SY2637, SY2640, SY2666, and SY2694 respectively and selection on SD-Leu.

Growth conditions

All strains were grown in rich medium supplemented with 3mM adenine (YPAD), unless otherwise specified. Unless otherwise indicated, cultures were grown in a shaking incubator at 30°C and maintained at an OD₆₀₀ of less than 0.5 for at least 10 doublings to ensure exponential growth.

Protein Analysis

SDS-PAGE and quantitative immunoblotting were performed as previously described (Pezza et al., 2009). Semi-Denaturing Detergent Agarose Electrophoresis (SDD-AGE) was performed as previously described (Kryndushkin et al., 2003).

Propagon Counts

The number of propagons per cell was determined by an *in vivo* colony-based dilution assay, as previously described (Cox et al., 2003). For propagon recovery experiments, cultures were first grown in YPAD + 3mM GdnHCl for 12 hours. Then, cells were pelleted, resuspended in YPAD to an OD of 0.1, and grown at 30°C. The number of propagons per cell was then determined at the indicated timepoints using the colony-based dilution assay.

Conversion Assay

Cultures were grown in SD +2.5mM adenine overnight, collected by centrifugation and incubated in medium conditioned by cells of the opposite mating type for one hour. Equal ODs of each mating partner were then mixed and incubated on solid SD + 2.5mM adenine and allowed to mate for 4 hours at 30°C (except where indicated, mating took place on solid SD + 2.5mM adenine + 3mM GdnHCl). Cells were then resuspended in SD + 2.5mM adenine and transferred to microscope slides for imaging.

Imaging

Imaging was performed in complete minimal medium supplemented with 2.5mM adenine and 2% glucose. Where indicated, medium contained 100µg/mL cycloheximide.

Static images were obtained on a Zeiss Axio Imager M2 fluorescent light microscope with a 100x objective. Microfluidics were performed on a Zeiss Axio Observer Z1 using a CellAsics microfluidics plate with temperature controls and media flow of 2 psi on a Y0C4 yeast perfusion plate (channel size 3.5-5µm). Fluorescence intensity was analyzed using the Zen software package (Zeiss, Germany).

Dual Luciferase Experiments

To measure luminescence, 10µL of yeast culture in early-log phase was added to 100µL passive lysis buffer (Promega) and incubated at room temperature for 2 minutes. Firefly and *Renilla* luciferase activity was then determined using the Dual-Luciferase Reporter Assay System and measured using a GloMax® 20/20 luminometer according to the manufacturer's instructions (Promega).

For luciferase recovery experiments, cultures were grown to an OD of 0.1 at 30°C, then incubated at 37°C for 30 minutes to induce chaperone expression. Cultures were then incubated at 40°C for 25 minutes, followed by addition of cycloheximide to 100 µg/mL, and returned to 40°C for 10 minutes, followed by recovery at 30°C. Dual luciferase assay or microscopy was then performed at the indicated timepoints.

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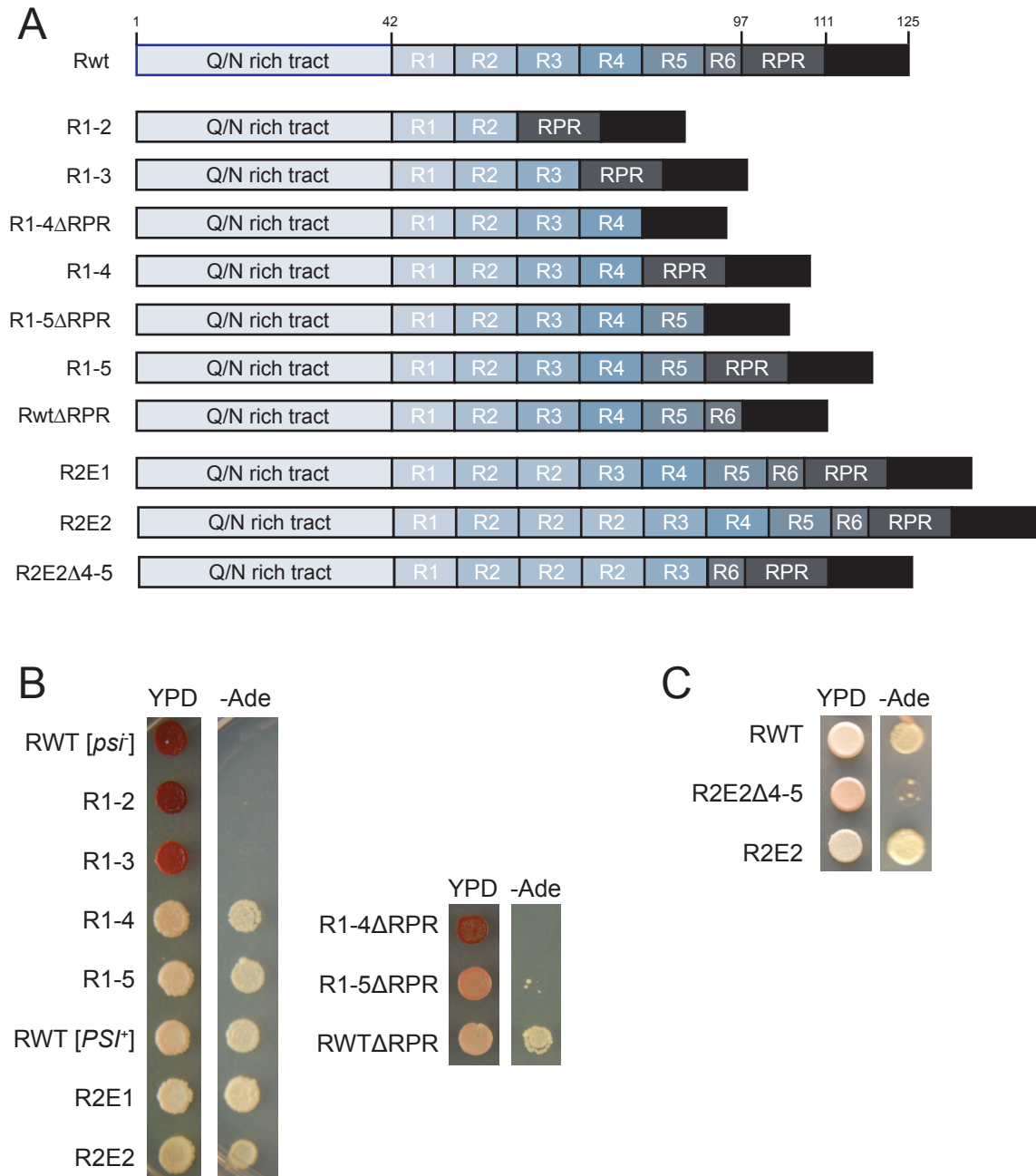


Figure 1. $[PSI^+]$ maintenance is dependent on composition of the repeat region. **A.** Schematic diagram of repeat variant constructs. **B.** Repeat variant strains (R1-2 (SY2072), R1-3 (SY2074), R1-4 (SY2057), R1-5 (SY2022), R2E1 (SY2247), R2E2 (SY2300), R1-4 Δ RPR (SY1629), R1-5 Δ RPR (SY1633), and Δ RPR (SY2023)) were spotted onto rich medium (YPD), or medium lacking adenine (-Ade) to analyze the $[PSI^+]$ phenotype. Wildtype $[psi^-]$ and $[PSI^+]$ strains (SLL2119 and SLL2606, respectively) are shown as controls. **C.** Repeat variant strains (WT (SLL2606), R2E2 Δ 4-5 (SY2808), and R2E2 (SY2300)) are spotted on rich medium (YPD), or media lacking adenine (-Ade) to analyze the $[PSI^+]$ phenotype.

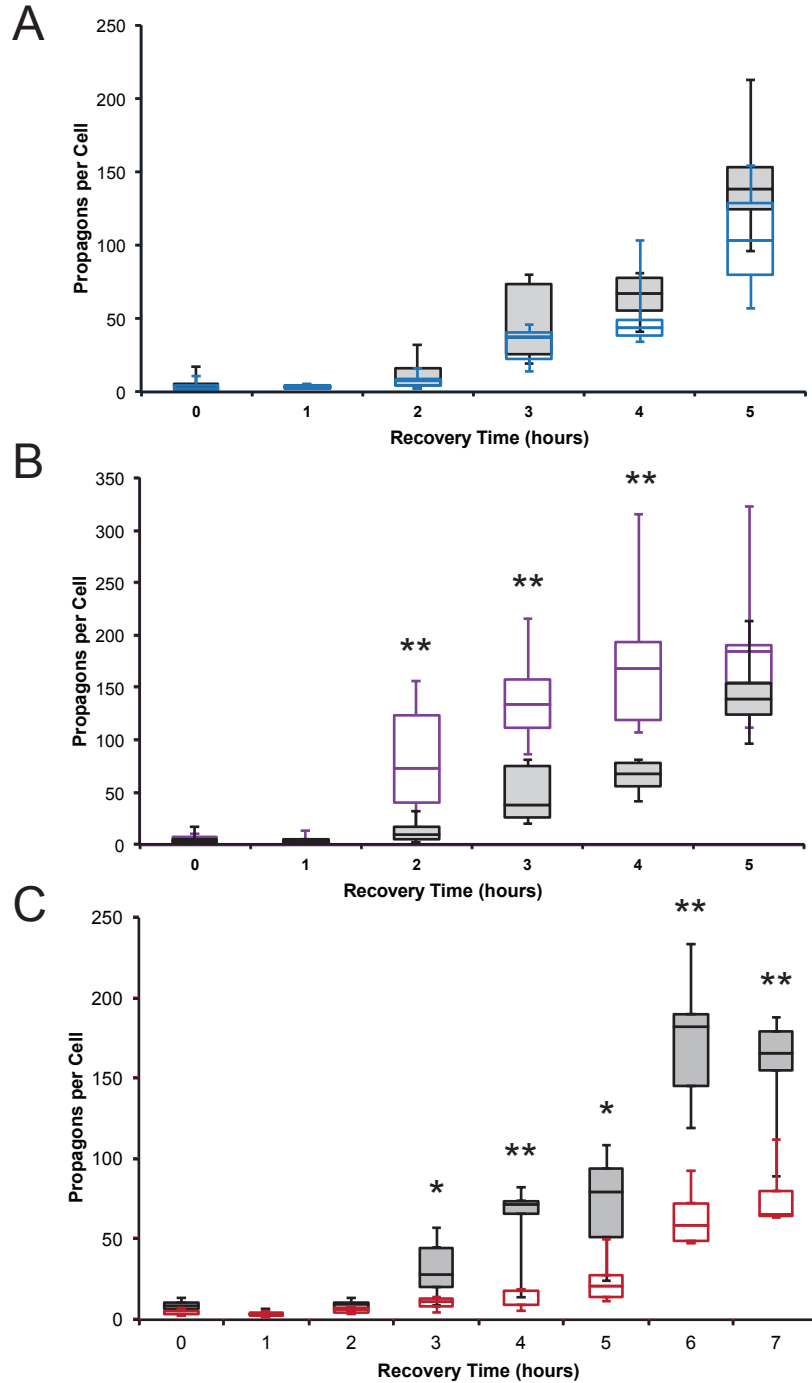


Figure 2. Repeat variant strains differ in their ability to amplify prion aggregates. **A.** Wildtype [*PSI*⁺] (gray, SLL2606) and R1-5 (blue, SY2022) strains were grown in YPAD + 3mM GdnHCl until nearly cured, followed by growth in YPAD for the indicated times. At each time point, the number of propagons per cell was determined. $n \geq 4$. Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles. Error bars indicate maximum and minimum. **B.** Wildtype [*PSI*⁺] (gray, SLL2606) and R2E1 (purple, SY2247) strains treated as in A. $n \geq 4$, ** $p < 0.01$, students t-test. **C.** Wildtype [*PSI*⁺] (gray, SLL2606) and Δ RPR (red, SY2023) strains were treated as in a. $n \geq 4$, * $p < 0.05$, ** $p < 0.01$, student's t-test.

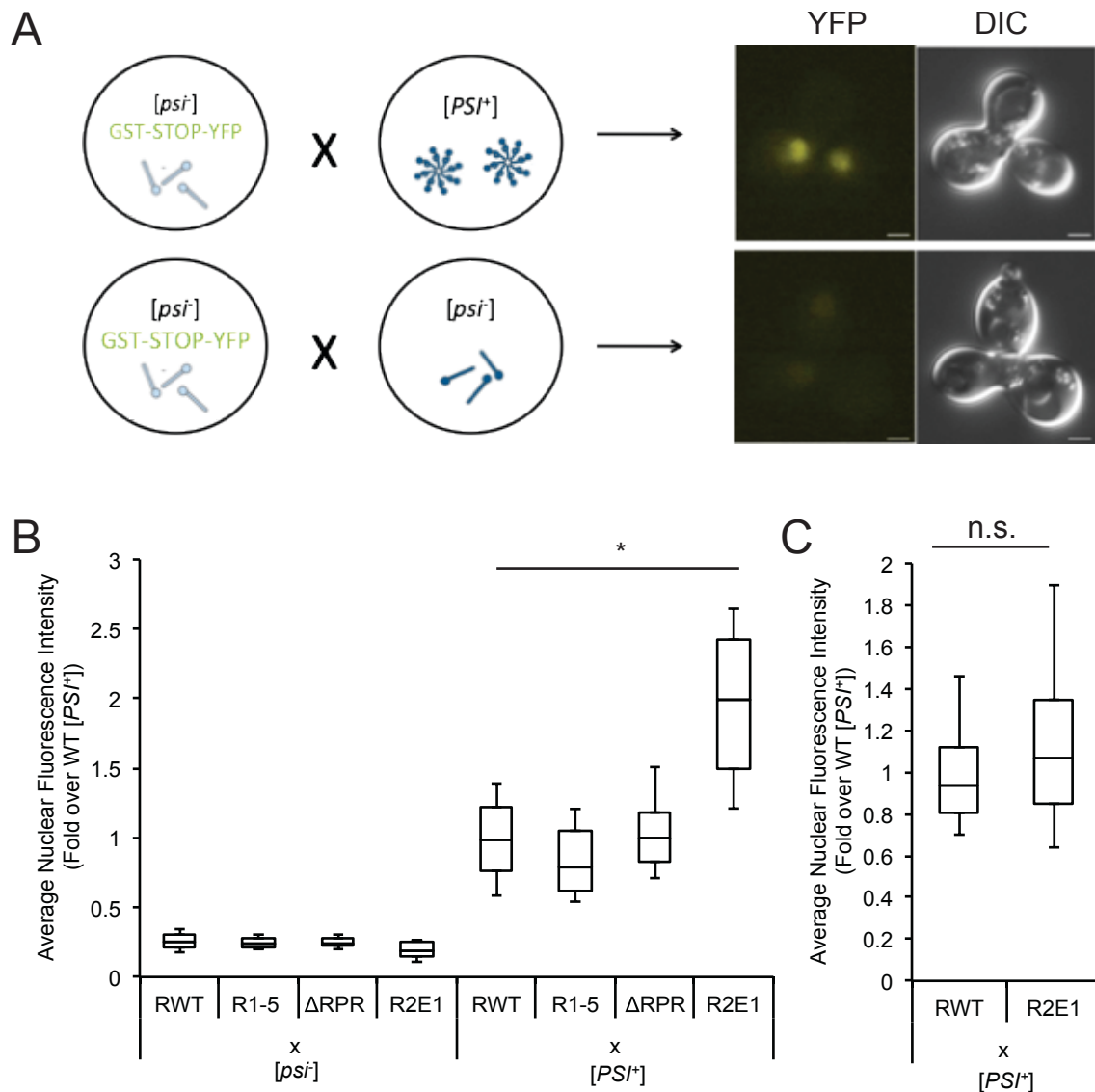


Figure 3. Neither repeats nor the RPR play a direct role in prion conversion.
A. Schematic representation of conversion mating assay. Ball and sticks represent soluble Sup35; pinwheels represent aggregated Sup35. **B.** Wildtype (SY2393), R1-5 (SY2461), Δ RPR (SY2463), and R2E1 (SY2465) $[\psi^-]$ strains containing the read-through reporter GST(UGA)YFP-NLS were mated to wildtype $[\psi^-]$ or $[\text{PSI}^+]$ (SLL2119 and SLL2606, respectively). Nuclear fluorescence intensity of unbudded zygotes was quantified. Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles. Error bars indicate 10th and 90th percentile. $n \geq 21$, $*p = 10^{-23}$, student's t-test. **C.** Wildtype (SY2393) and R2E1 (SY2465) strains containing the read-through reporter GST(UGA)YFP-NLS were mated to wildtype $[\text{PSI}^+]$ (SLL2606) in the presence of guanidine HCl. Nuclear fluorescence intensity of unbudded zygotes was determined. Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles. Error bars indicate 10th and 90th percentile. $n \geq 38$, n.s. not significant.

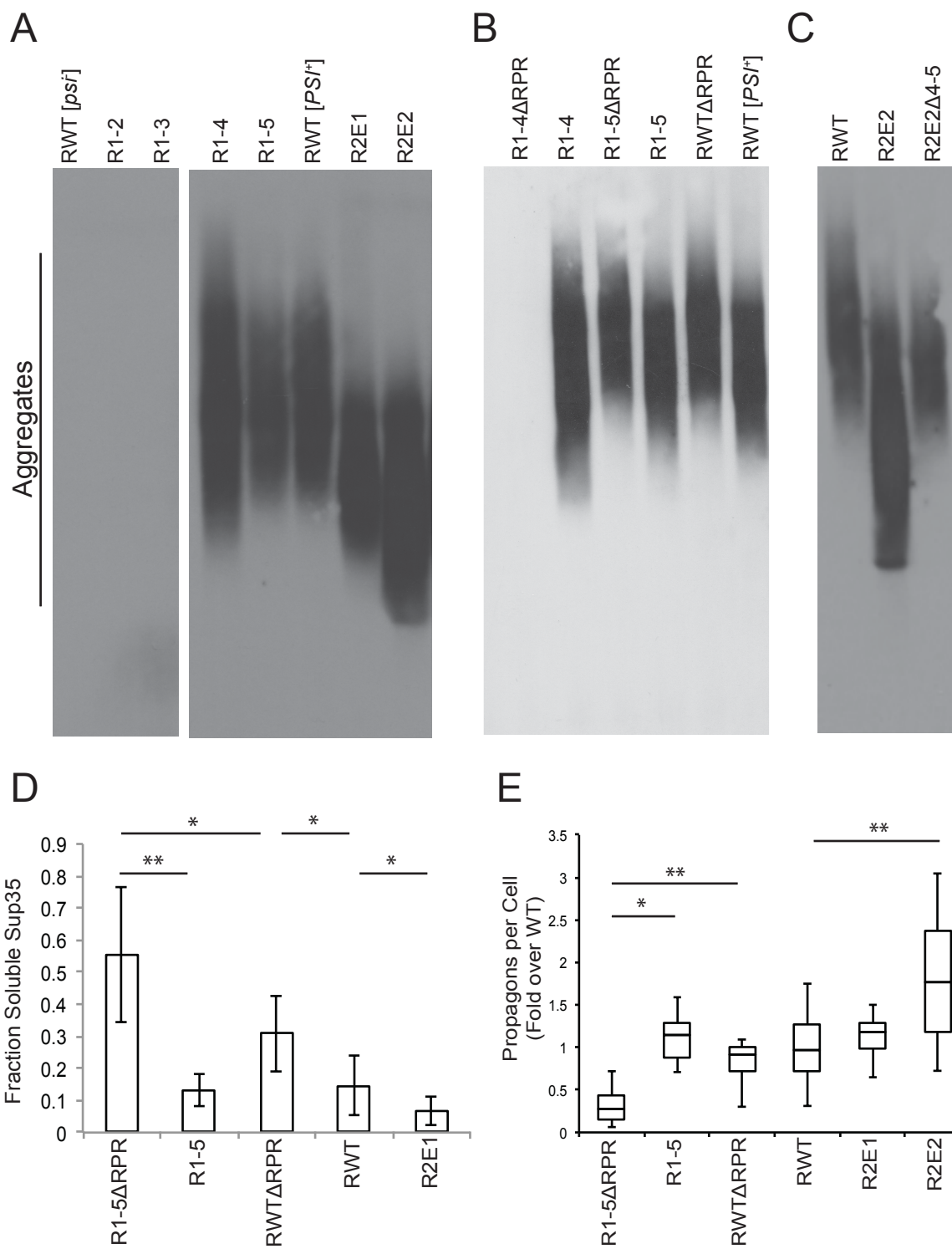


Figure 4. Repeats and RPR promote the fragmentation of prion aggregates.

a. SDD-AGE was performed on wildtype [*psi*⁻] (SLL2119), R1-2 (SY2072), R1-3 (SY2074), R1-4 (SY2057), R1-5 (SY2022), wildtype [*PSI*⁺] (SLL2606), R2E1 (SY2247), R2E2 (SY2300) lysates, followed by immunoblot for Sup35. **B.** SDD-AGE was performed on R1-4ΔRPR (SY1629), R1-4 (SY2057), R1-5ΔRPR (SY1633), R1-5 (SY2022), ΔRPR (SY2023), and wildtype [*PSI*⁺] (SLL2606) lysates, followed by immunoblot for Sup35. **C.** SDD-AGE was performed on wildtype [*PSI*⁺] (SLL2606), R2E2 (SY2300), and R2E2Δ4-5 (SY2808) lysates, followed by immunoblot for Sup35. **D.** Cell lysates from R1-5ΔRPR (SY1633), R1-5 (SY2022), ΔRPR (SY2023), wildtype [*PSI*⁺] (SLL2606) and R2E1 (SY2247) were incubated at 53°C and 100°C in the presence of SDS before SDS-PAGE and immunoblotting for Sup35. The amount of soluble Sup35 was determined as the percentage of signal at 53°C relative to 100°C. Bars represent means, error bars represent standard deviations, n≥5, *p<0.05, **p<0.01, student's t-test. **E.** The number of propagons in R1-5ΔRPR (SY1633), R1-5 (SY2022), ΔRPR (SY2023), wildtype [*PSI*⁺] (SLL2606), R2E1 (SY2247), and R2E2 (SY2300) were determined by an *in vivo* colony based dilution assay. Horizontal lines on boxes represent the 25th, 50th, and 75th percentiles. Error bars represent maximum and minimums. n≥13, *p<0.05, **p<0.01, student's t-test.

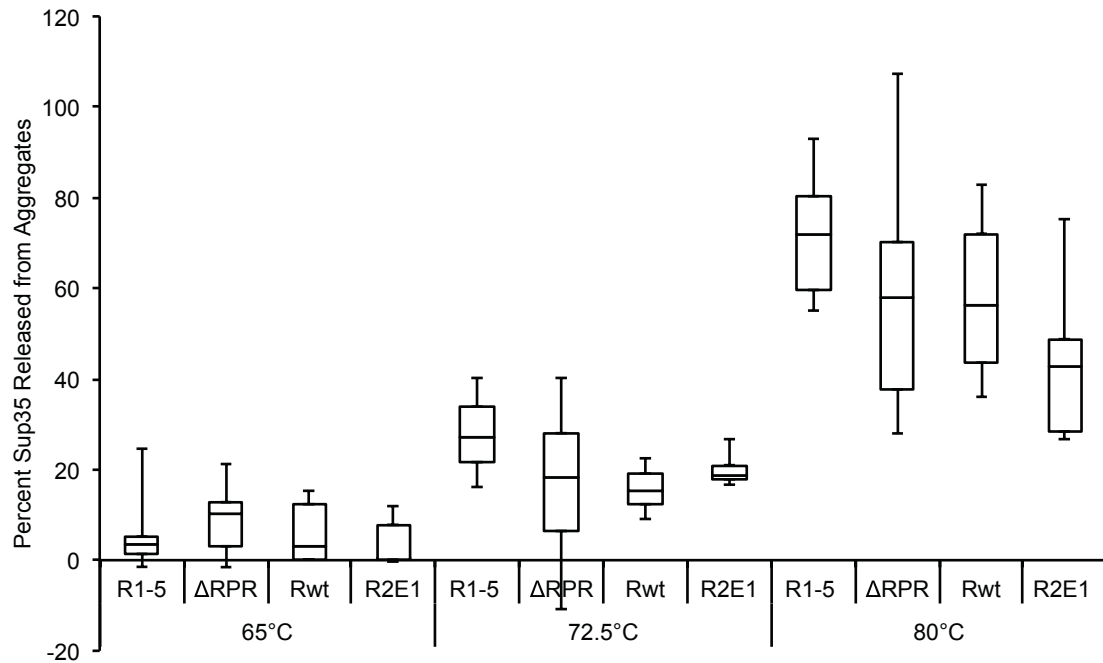


Figure 5. Repeat number does not affect aggregate thermodynamic stability. Lysates from R1-5 (SY2022), Δ RPR (SY2023), wildtype (SLL2606), and R2E1 (SY2247) strains were incubated in SDS at the indicated temperatures before SDS-PAGE. The percent of Sup35 released from aggregates at each temperature was determined relative to an incubation at 53°C. Horizontal lines on boxes represent 25th, 50th, and 75th percentiles. Error bars represent maximums and minimums. $n \geq 3$.

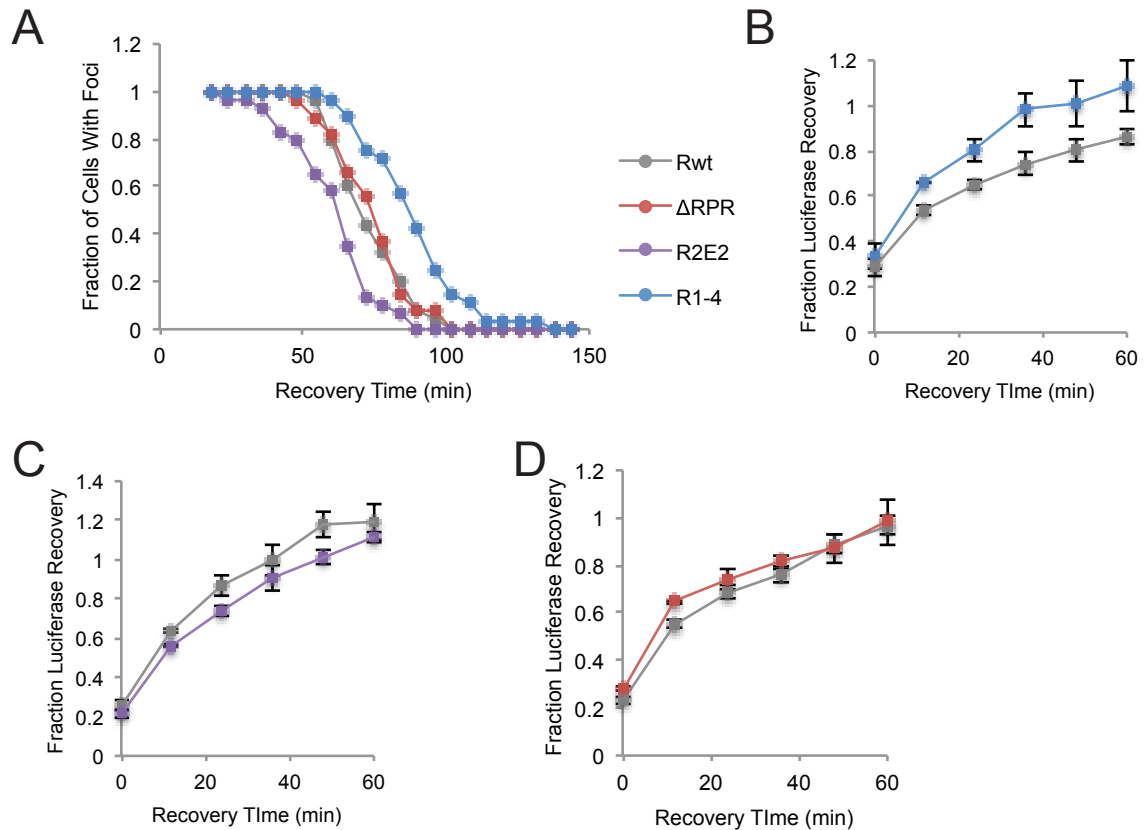


Figure 6. Repeat number and RPR differently affect luciferase disaggregation and refolding following heat shock.

A. The indicated strains expressing luciferase reporter constructs were incubated at 37°C for 30 minutes, followed by 40°C for 35 minutes, with cycloheximide added for the last 10 minutes, in a microfluidics chamber. Cells were then allowed to recover at 30°C in the presence of cycloheximide, and images were taken at the indicated time points. At each time point, the percentage of cells containing GFP foci was determined. **B.** Strains expressing luciferase reporter constructs containing Sup35NWT (SY2393, grey) or Sup35NR1-4 (SY2666, blue) were treated as in A. At each time point, the level of firefly luciferase activity was determined. Error bars represent standard deviation, n=3. **C.** Strains expressing luciferase reporter constructs containing Sup35NWT (SY2393, grey) or Sup35NR2E2 (SY2640, purple) were treated as in A. At each time point, the level of firefly luciferase activity was determined. Error bars represent standard deviation, n=3. **D.** Strains expressing luciferase reporter constructs containing Sup35NWT (SY2393, grey) or Sup35N Δ RPR (SY2637, red) were treated as in A. At each time point, the level of firefly luciferase activity was determined. Error bars represent standard deviation, n=3.

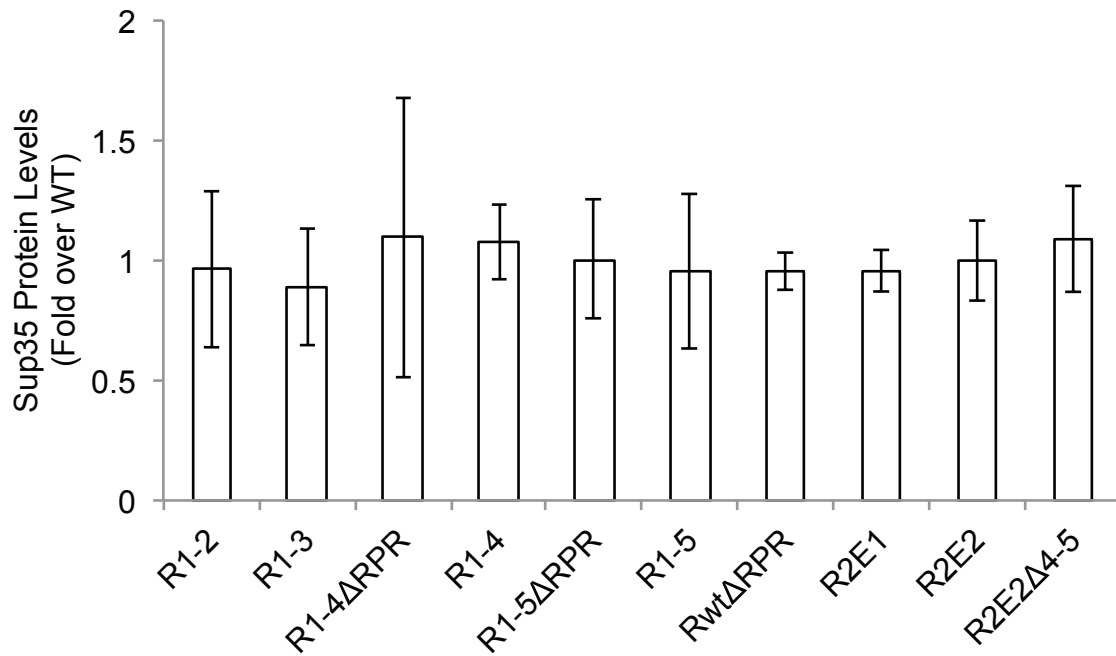


Figure S1. Sup35 expression levels are similar in all repeat variant strains. Lysates from R1-2 (SY2072), R1-3 (SY2073), R1-4ΔRPR (SY1629), R1-4 (SY2057), R1-5ΔRPR (SY1633), R1-5 (SY2022), ΔRPR (SY2023), R2E1 (SY2247), R2E2, SY2300 and R2E2Δ4-5 (SY2808) were analyzed by SDS-PAGE and quantitative immunoblotting for Sup35. Bars represent means, error bars represent standard deviations. $n \geq 3$.

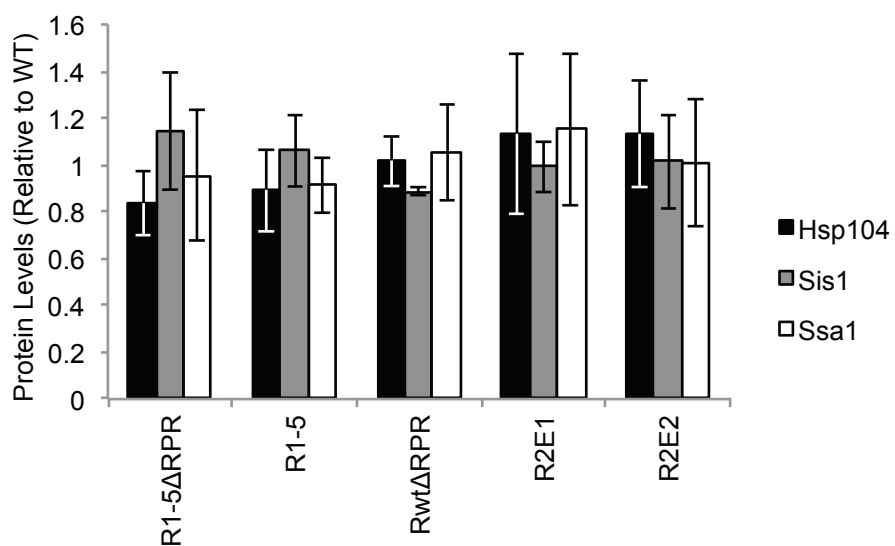


Figure S2. Chaperone levels are similar in repeat variant strains. R1-5ΔRPR (SY1633), R1-5 (SY2022), ΔRPR (SY2023), R2E1 (SY2247), R2E2 (SY2300) were analyzed by SDS-PAGE and quantitative immunoblotting for Hsp104 (black), Ssa1 (gray), and Sis1(white). Bars represent means, error bars represent standard deviations. n≥3.

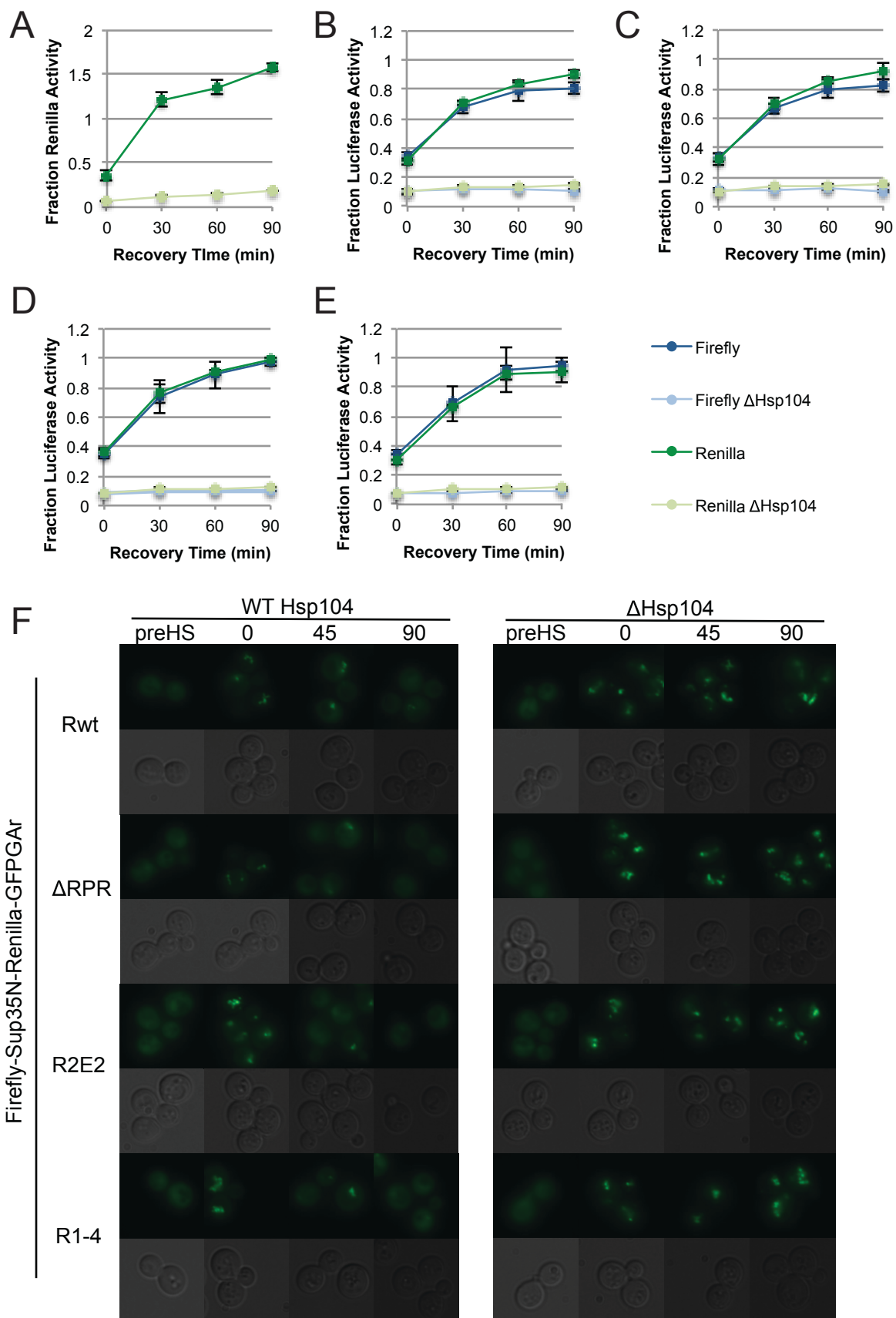


Figure S3. Luciferase recovery following heat shock is dependent on Hsp104.

A. Strains containing Renilla luciferase-GFP with or without an Hsp104 disruption (SY2694 and SY2703, respectively) were heat shocked at 37°C for 30 min, followed by 40°C for 35 minutes, with cycloheximide added for the last 10 minutes and recovery at 30°C in the presence of cycloheximide. Renilla luciferase activity was measured at the indicated time points. n=3, data represent means, error bars indicate standard deviations. **B.** Strains containing Firefly luciferase-Sup35N-Renilla luciferase-GFP with or without an Hsp104 disruption (SY2603 and SY2625, respectively) were treated as in A. Firefly and renilla luciferase activity was measured at the indicated time points. n=3, data represent means, error bars indicate standard deviations. **C.** Strains containing Firefly luciferase-Sup35NR1-4-Renilla luciferase-GFP with or without an Hsp104 disruption (SY2666 and SY2679, respectively) were treated as in A. Firefly and renilla luciferase activity was measured at the indicated time points. n=3, data represent means, error bars indicate standard deviations. **D.** Strains containing Firefly luciferase-Sup35NΔRPR-Renilla luciferase-GFP with or without an Hsp104 disruption (SY2637 and SY2651, respectively) were treated as in A. Firefly and renilla luciferase activity was measured at the indicated time points. n=3, data represent means, error bars indicate standard deviations. **E.** Strains containing Firefly luciferase-Sup35NR2E2-Renilla luciferase-GFP with or without an Hsp104 disruption (SY2640 and SY2654, respectively) were treated as in A. Firefly and renilla luciferase activity was measured at the indicated time points. n=3, data represent means, error bars indicate standard deviations. **F.** Strains containing Firefly luciferase-Sup35N(RV)-Renilla luciferase-GFP with or without an Hsp104 disruption were treated as in A and imaged at the indicated time points. Representative images are shown.

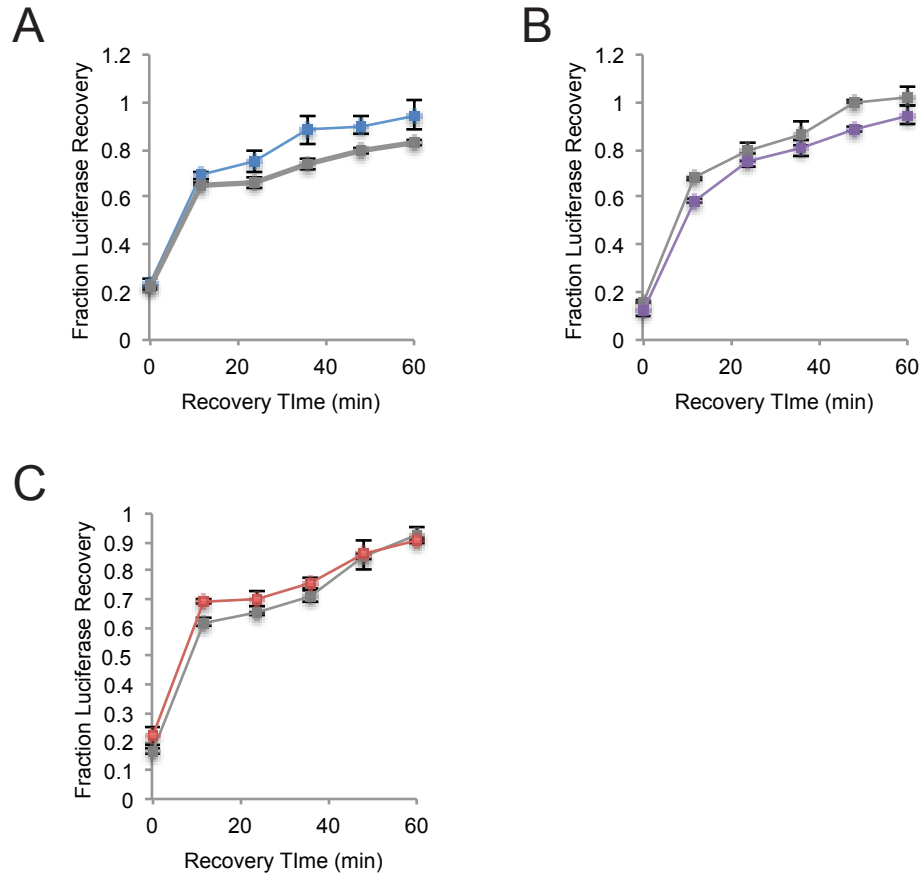


Figure S4. Repeat number affects renilla luciferase refolding following heat shock.

A. Strains containing luciferase reporter constructs containing Sup35NWT (SY2393, grey) or Sup35NR1-4 (SY2666, blue) were heat shocked as in A. At each time point, the level of renilla luciferase activity was determined. Error bars represent standard deviation, n=3. **B.** Strains containing luciferase reporter constructs containing Sup35NWT (SY2393, grey) or Sup35NR2E2 (SY2640, purple) were heat shocked as in A. At each time point, the level of renilla luciferase activity was determined. Error bars represent standard deviation, n=3. **C.** Strains containing luciferase reporter constructs containing Sup35NWT (SY2393, grey) or Sup35NΔRPR (SY2637, red) were heat shocked as in A. At each time point, level of renilla luciferase activity was determined. Error bars represent standard deviation, n=3.

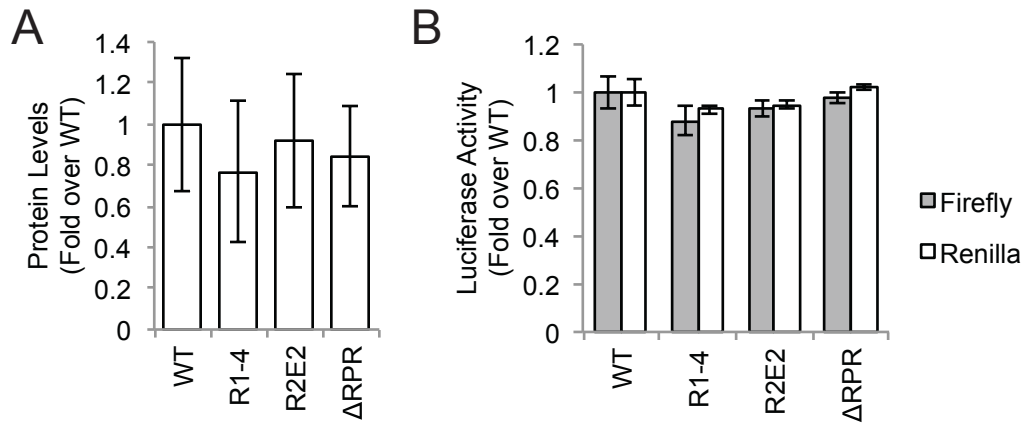


Figure S5. Expression and activity of dual luciferase reporter constructs are similar

A. Lysates from strains containing Firefly-Sup35N-Renilla-GFP reporters were analyzed by SDS-PAGE and quantitative immunoblotting for GFP. $n=3$, bars represent means, error bars represent standard deviations. **B.** The levels of firefly and renilla luciferase activity were determined in strains expressing the indicated reporters. $n=3$, bars represent means, error bars represent standard deviations.

Chapter 3:
**Effects of Amyloid Aggregate Fragmentation on Prion Appearance and
Toxicity**

I performed all of the experiments in this chapter.

Abstract

Prion phenotypes are stabilized *in vivo* through a multi-step process. First, protein in the non-prion form must be efficiently converted to the prion state. Second, the resulting prion aggregates must be efficiently fragmented by molecular chaperones, a step that both creates new templates for conversion and ensure efficient inheritance of prion aggregates to new cells. Importantly, maintaining a precise rate of fragmentation is critically important to maintain the prion state, as either increasing or decreasing chaperone levels leads to loss of the prion phenotype. Here, we use a set of repeat variants of the Sup35/[PSI⁺] prion to investigate the role of fragmentation in other aspects of prion biogenesis, specifically prion induction and cellular toxicity. We show that both increases and decreases in fragmentation increase prion induction and result in cellular toxicity. Our results highlight the essential and central role of fragmentation in prion biogenesis.

Introduction

To remain viable, cells must maintain a properly folded proteome. Amyloidogenic proteins, however, are prone to misfolding and form β sheet-rich aggregates (Chiti and Dobson, 2006; Jahn and Radford, 2008). These proteins include those implicated in a variety of neurodegenerative disease, including Alzheimer's, Parkinson's, and Huntington's disease, as well as the prion diseases (Chiti and Dobson, 2006). To protect itself from misfolded aggregates, the cell employs a network of quality control processes, including those involved in refolding and degradation of misfolded species (Frydman, 2001). However, it is possible for these quality control systems to fail or become overwhelmed, leading to the build up of protein aggregates, which can result in toxicity. In contrast, yeast prions are non-pathogenic and therefore serve as an amenable system for studying the interaction between aggregation and the cellular response to these aberrant species. Specifically, the Sup35 protein can exist in a properly folded, soluble state ($[psi^-]$), or a variety of misfolded and aggregated states ($[PSI^+]$) (Cox, 1965; Patino et al., 1996; Paushkin et al., 1996). $[PSI^+]$ can arise spontaneously at a low frequency ($\sim 10^{-7}$) *in vivo* (Lancaster et al., 2010; Liu and Lindquist, 1999). In addition, the appearance of $[PSI^+]$, as well as other yeast prions, is greatly enhanced by overexpression of the determinant protein (Chernoff et al., 1993; Derkatch et al., 1996), consistent with the idea that increased expression promotes protein misfolding. In addition, the formation of prion aggregates may require an imbalance between misfolded proteins and the cellular mechanisms in place to clear them.

Once they arise, prions and other amyloid aggregates alter cellular phenotypes by changing the activity of the aggregated protein through one of two main pathways. First, the aggregation of proteins can result in a loss-of-function phenotype, as is the case for the yeast prions Sup35 and Ure2. Sup35 is an essential component of the translation termination machinery, and its aggregation in the prion ($[PSI^+]$) form leads to stop codon read-through (Cox, 1965; Ter-Avanesyan et al., 1994). Similarly, Ure2 is responsible for nitrogen catabolite repression, and its aggregation in the prion ($[URE3]$) form allows yeast to constitutively use poor nitrogen sources (Wickner, 1994). Second, the aggregation of proteins can result in a gain-of-function phenotype, as is the case for the yeast prion Rnq1 and for mammalian proteins with glutamine expansions (polyQ). Rnq1 is a protein of unknown function, but its aggregation in the prion ($[RNQ^+]$) form allows for the induction of other prions, including $[PSI^+]$ and $[URE3]$ (Derkatch et al., 1997, 2000, 2001). In mammals, aggregation of polyQ proteins leads to toxicity, likely through the sequestration of other essential proteins that contain glutamine-rich stretches (Kim et al., 2002; Olzscha et al., 2011). These aberrant interactions have been shown to include both CREB binding protein and TATA binding protein (Chai et al., 2002; McCampbell et al., 2000; Perez et al., 1998; Steffan et al., 2000). In addition, non-toxic amyloid aggregates, such as $[PSI^+]$ and $[RNQ^+]$ can also lead to cellular toxicity upon overexpression of Sup35 or Rnq1 respectively through a similar sequestration mechanism (Treusch and Lindquist, 2012; Vishveshwara et al., 2009).

Prions form dynamic protein complexes that are stable and transmissible. To persist, prion complexes must grow through conversion and incorporation of newly made, soluble protein to the prion state (Satpute-Krishnan and Serio, 2005). These resulting complexes are then amplified through fragmentation by a molecular chaperone, which creates new templates for continued conversion (Chernoff et al., 1995; Satpute-Krishnan et al., 2007; Serio et al., 2000). In yeast, fragmentation is carried out by the AAA+ ATPase Hsp104 and its co-chaperones Ssa1 (Hsp70) and Sis1 (Hsp40) (Chernoff et al., 1995; Higurashi et al., 2008; Satpute-Krishnan et al., 2007; Tipton et al., 2008). When Hsp104 is deleted or inhibited, prions cannot arise *de novo*, and existing prions are lost (Chernoff et al., 1995; Ness et al., 2002). Overexpression of Hsp104 alone or in combination with Ssa1 and Sis1 also results in prion loss, likely due to an imbalance with other chaperones in the cell that leads to non-productive interactions between prion aggregates and the cellular machinery responsible for their propagation (Chernoff et al., 1995; Winkler et al., 2012). In addition, Hsp104, Ssa1 and Sis1 are also required for the appearance and propagation of polyQ amyloid aggregates in yeast, and overexpression of chaperones in this context reverses the toxicity associated with these complexes (Krobitsch and Lindquist, 2000; Park et al., 2013). Together, these data indicate that precise levels of fragmentation are required for both amyloid appearance and maintenance within the cell.

For the Sup35/[PSI⁺] prion, specific sequences mediate prion aggregate fragmentation. The Sup35 prion-forming domain (NM, amino acids 1-254), which

is required for its incorporation into amyloid aggregates but dispensable for its function in translation termination, is composed of a glutamine/asparagine (Q/N)-rich tract, which plays a role in conversion, followed by five and a half imperfect oligopeptide repeats. Deletion of repeats results in prion loss, while repeat expansion results in an increase in the spontaneous appearance of $[PSI^+]$ (Liu and Lindquist, 1999). We have proposed that the repeat region acts to promote fragmentation, with a decrease in repeat number being associated with a decrease in fragmentation, and an increase in repeat number being associated with an increase in fragmentation (see Chapter 2). Here, we use strains containing Sup35 repeat variants to explore whether changing Sup35 prion aggregate dynamics via modulation of fragmentation affects prion appearance and/or toxicity.

Results and Discussion

Effect of fragmentation on prion induction

The precise mechanism by which prions arise *in vivo* remains unclear. The dominant model in the field suggests that a heterologous template is required to initiate prion conversion (Derkatch et al., 2001; Osherovich and Weissman, 2001). However, several pieces of data have implicated the chaperone machinery in playing a crucial role in this process. Specifically, transient overexpression of Hsp104 decreased the appearance of $[PSI^+]$ following Sup35 overexpression, but produced a greater percentage of less

thermodynamically stable (i.e. more easily fragmented) variants (Borchsenius et al., 2006; Kryndushkin et al., 2011), suggesting that an increase in fragmentation during *de novo* induction might inhibit prion formation, possibly because aggregates are dissolved before they can be replicated. Similarly when Hsp104 levels are increased as a result of an N-terminal deletion in heat shock factor 1 (HSF1), *de novo* [*PSI*⁺] induction is decreased (Park et al., 2006). In contrast when Hsp104 levels are decreased, [*PSI*⁺] *de novo* induction is enhanced (Villali and Serio, unpublished). In addition, when other chaperone co-factors (i.e. Hsp90 Sti1 and Hsp70 Sse1) levels are lowered as a result of a C-terminal deletion in HSF1, *de novo* [*PSI*⁺] induction is enhanced (Park et al., 2006), and this effect is consistent with the observation that Sti1 antagonizes prion formation (Jones et al., 2004). Together, these data suggest that a precise level of fragmentation by Hsp104 and its co-chaperones is required for prion induction.

Here, we use strains expressing Sup35 containing different numbers of repeats as a model for how changing fragmentation affects the *de novo* induction of [*PSI*⁺]. We transiently overexpressed a fusion between the Sup35 prion determining domain (NM) containing 3, 4, 5, and 5.5 (wildtype) repeats and the green fluorescent protein (GFP) in a [*psi*⁻] [*RNQ*⁺] strain expressing endogenous wildtype Sup35. Consistent with our previous observations of enhanced prion appearance in strains with reduced Hsp104 activity (Villali and Serio, unpublished), [*PSI*⁺] induction was significantly increased when the R1-5 variant fusion, which is fragmented with reduced efficiency (see Chapter 2), was expressed compared to wildtype (Figure 1). However, when fragmentation was

decreased further, as in the R1-3 and R1-4 strains, we observed decreased $[PSI^+]$ formation (Figure 1). This trend supports a model in which specific levels of fragmentation are required for prion appearance. When wildtype Sup35 is present, a competition between protein misfolding/aggregation and clearance by Hsp104 exists. Slightly lowering the frangibility of aggregates, as in the R1-5 variant, provides an opportunity for aggregates to grow sufficiently large to survive the fragmentation event, leading to their amplification rather than their clearance. However, further decreases in fragmentation no longer support $[PSI^+]$ appearance, possibly because these nascent aggregates cannot be sufficiently amplified prior to cell division to persist in the population.

Consistent with this interpretation, mice expressing PrP lacking the repeat region (PrP Δ OR) are susceptible to disease but have longer incubation times and lower prion titers in the brain than mice expressing wildtype PrP (Flechsiger et al., 2000; Yamaguchi et al., 2012). In addition, PrP Δ OR containing the repeat deletion remained protease resistant (Yamaguchi et al., 2012), suggesting that the delay in disease onset is not due to a decreased ability of PrP Δ OR to misfold. This observation is consistent with our studies in yeast, which indicate that Sup35 variants lacking a sufficient number of repeats accumulate fewer heritable prion aggregates (propagons, see Chapter 2).

Interestingly, the repeat expansion R2E2 has previously been shown to increase the rate of spontaneous appearance of $[PSI^+]$ about 100-fold (Liu and Lindquist, 1999), an observation which is counter to the evidence that increased fragmentation should decrease induction frequency. However, the repeat

expansions do contain an increase in Q/N residues, and upon their overexpression, may have an increased propensity to misfold independently of cellular factors. Consistent with this idea, the rate of spontaneous amyloid formation for repeat expansions *in vitro*, which occurs independent of chaperones, is accelerated relative to wildtype (Hess et al., 2007; Liu and Lindquist, 1999). Thus, the higher induction rate of the repeat expansion strains may not be attributed to a change in the protein's interaction with cellular factors but rather to a change in the physical properties of Sup35 itself.

Mice expressing PrP with a repeat expansion from 5 to 14 (PrP14) repeats have an earlier age of onset of disease, as compared to wildtype (Chiesa et al., 2000). However, PrP14 mice also show a 10-fold increase in accumulation of PrP protein compared to wildtype mice (Chiesa et al., 2000), suggesting that the increased induction in this case may be due to protein accumulation and/or resistance to degradation. In addition, familial cases of prion disease associated with repeat expansions have an earlier age of onset (Guo et al., 2008; Jansen et al., 2009; Mauro et al., 2008), consistent with an increased propensity for repeat expanded PrP to misfold.

Effect of fragmentation on cellular toxicity

[PSI⁺] and other yeast prions are not normally toxic at their endogenous expression levels. However, we noticed that some of our repeat variant strains had a slow growth phenotype (Figure 2, dark grey bars). The toxic strains included a repeat deletion (R1-4, containing 4 repeats), and both repeat

expansions (R2E1 and R2E2 containing 6.5 and 7.5 repeats, respectively), with the effect being most severe in R2E2. Additionally, toxicity was dependent on the $[PSI^+]$ state, as guanidine-cured repeat variant strains had no growth defect (Figure 2, light grey bars). Interestingly, these toxic strains were those with the lowest and highest levels of fragmentation (see Chapter 2), suggesting that toxicity may be correlated to aberrant levels of fragmentation. Consistent with this idea, the expression of Hsp104 is elevated in the $[PSI^+]$, but not the $[psi^-]$ R1-4 strain (Figure 3).

One well-characterized manipulation that can lead to $[PSI^+]$ toxicity is overexpression of either the prion domain of Sup35 (Sup35NM) or of full-length Sup35. Toxicity induced by overexpression of Sup35NM is relieved by co-overexpression of the functional domain of Sup35 (Sup35C) (Vishveshwara et al., 2009). Because Sup35C lacks the prion domain, it is unable to join prion aggregates and remains soluble and functional (Ter-Avanesyan et al., 1994). Its ability to rescue Sup35NM overexpression-induced toxicity suggests that the toxicity can be attributed to a loss of functional Sup35, as all of the full-length Sup35 has been sequestered into aggregates. In addition, toxicity upon overexpression of Sup35NM is associated with a loss of smaller prion aggregates, which also contribute to translation termination levels, further promoting translational readthrough of stop codons (Pezza et al., 2014). However, smaller prion aggregates were not lost in any of the toxic strains (Chapter 2, Figure 4A). On the contrary, the Sup35 aggregates in the repeat expansion strains were even smaller than those found in a wildtype strain

(Chapter 2, Figure 4A), suggesting that a loss of functionally engaged aggregates is not responsible for the toxicity observed in these strains. However, the repeat expansion strains have reduced soluble Sup35 compared to wildtype (~15% soluble), with R2E2 (soluble undetectable) having even less steady-state soluble Sup35 than R2E1 (~6% soluble) (Chapter 2, Figure 4D), suggesting that toxicity in these strains may be attributed to a loss of soluble and functional Sup35. To test this hypothesis, we expressed an extra copy of Sup35C in these strains. As expected, expressing an extra copy of C in the repeat expansion strains was sufficient to relieve the toxicity, returning the growth rate to wild-type levels (Figure 2, white bars). Surprisingly, expressing an extra copy of Sup35C also relieved the toxicity in the R1-4 strain (Figure 2, white bars), despite the fact that this strain does not have a decreased level of steady-state soluble Sup35 (R1-4 has ~18% soluble) compared to wildtype (Chapter 2, Figure 4D), suggesting that aggregates in this strain must sequester another essential cofactor that interacts with Sup35.

Indeed, toxicity induced by overexpression of full-length Sup35 can be relieved by co-overexpression of Sup45 (Tank and True, 2009; Vishveshwara et al., 2009). Sup45 (eRF1) is an essential protein that functions with Sup35 in translation termination, and its interaction with Sup35 is mediated by the C-terminal domain of Sup35. Thus, toxicity induced by overexpression of full-length Sup35 has been attributed to the sequestration of Sup45 into prion aggregates. Consistent with this model, Sup45 colocalizes with Sup35 aggregates in [*PSI*⁺] but not [*psi*⁻] cells overexpressing Sup35 (Vishveshwara et al., 2009), and Sup45

has been reported to co-sediment with Sup35 aggregates in lysates from some strains (Czaplinski et al., 1998; Paushkin et al., 1997; Pezza et al., 2014). Similarly, we propose that the lack of fragmentation in the R1-4 strain leads to sequestration of Sup45. More specifically, according to our model, Hsp104 is able to easily extract Sup35 monomers from R1-4 aggregates (see chapter 2). This easy extraction leads to less destabilization of the aggregates along with any cofactors that may be bound. Thus, once Sup45 binds to prion aggregates, the Sup35-Sup45 interaction in the R1-4 strain is more stable compared to wildtype. This stable binding reduces the amount of soluble Sup45 available for translation termination, thereby leading to toxicity. Consistent with this model, expression of an extra copy of Sup45 in the R1-4 strain reduces toxicity (data not shown). We propose that when an extra copy of Sup35C is expressed in these strains, the toxicity is relieved because soluble Sup35C is able to compete for Sup45 binding, titrating enough Sup45 away from aggregates that its function in translation termination is restored.

There are two models that have been proposed to explain amyloid toxicity in disease. The first model proposes that amyloid aggregates may be toxic themselves. However, several pieces of data contradict this model. Naturally occurring amyloids such as yeast prions are usually non-toxic, and some amyloids even make functional contributions, such as CsgA in *E. coli*, where amyloid assembly promotes biofilm formation (Chapman et al., 2002). In addition, there are not significant structural differences between toxic and benign amyloids (Baxa, 2008), suggesting that it is unlikely that a feature of the amyloid

itself is the toxic agent. In the second model, amyloids are not toxic *per se*, but the toxicity results from aberrant interactions between the amyloids and the cellular environment. Consistent with this model, quantitative proteomics of model amyloid aggregates revealed that the aggregate-induced toxicity correlated with the amyloid's ability to promote aberrant interactions with cellular proteins (Olzscha et al., 2011). Furthermore, several quality control factors, including Hsp70 and Hsp110 were sequestered into these aggregates, and overexpression of Hsp110 was sufficient to mitigate toxicity (Olzscha et al., 2011). In addition, toxicity of polyQ expanded huntingtin protein, which leads to the accumulation of aggregates in a glutamine length-dependent manner (Duennwald et al., 2006; Krobitch and Lindquist, 2000), in yeast is enhanced by the presence of $[RNQ^+]$ and $[PSI^+]$, and can be relieved by expression of Sup35C or overexpression of Sup45, suggesting that toxicity is a result of sequestration of essential cellular factors (Gong et al., 2012; Kochneva-Pervukhova et al., 2012; Zhao et al., 2012). Our data support this sequestration model, and also argue that the efficiency by which Sup35 prion aggregates are processed by molecular chaperones is an important determinant for this sequestration and subsequent toxicity.

Table 1: Plasmids

	Description
SB804	pRS306-P _{SUP35} Sup35R1-3
SB775	pRS306-P _{SUP35} Sup35R1-4
SB776	pRS306-P _{SUP35} Sup35R1-5
SB787	pRS306-P _{SUP35} Sup35R2E1
SB883	pRS303-P _{SUP35} Sup35R2E1
SB859	pRS306-P _{SUP35} Sup35R2E2
SB884	pRS303-P _{SUP35} Sup35R2E2
SLL6682	PRS306-P _{SUP35} C
SLL6442	pRS426-P _{CUP} Sup35NM-GFP
SB865	pRS426-P _{CUP} Sup35NM(R1-3)-GFP
SB866	pRS426-P _{CUP} Sup35NM(R1-4)-GFP
SB867	pRS426-P _{CUP} Sup35NM(R1-5)-GFP

Table 3: Strains

	Genotype	Plasmids Integrated	Reference
SLL2606	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112</i>	-	Chernoff et al. 1995
SLL2119	<i>MATa [psi⁻] [RNQ⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112</i>	-	Chernoff et al. 1995
SY2057	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-4</i>	SB775	This study
SY2247	<i>MATa [PSI⁺] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E1 trp1-289 ura3-52 leu2-3, 112 SUP35R2E1</i>	SB787, SB883	This study
SY2300	<i>MATa [PSI⁺] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E2 trp1-289 ura3-52 leu2-3, 112 sup35::P_{MFA1}SUP35R2E2::KANMX6</i>	SB859, SB884	This study
SY2073	<i>MATa [psi⁻] [RNQ⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-3</i>	SB804	This study
SY2213	<i>MATa [psi⁻] [RNQ⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-4</i>	SB775	This study
SY2215	<i>MATa [psi⁻] [RNQ⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35R1-5</i>	SB776	This study
SY2302	<i>MATa [psi⁻] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E1 trp1-289 ura3-52 leu2-3, 112 SUP35R2E1</i>	SB787, SB883	This study
SY2305	<i>MATa [psi⁻] ade1-14 his3Δ200::HIS3::P_{Sup35}SUP35R2E2 trp1-289 ura3-leu2-3, 112 sup35::P_{MFA1}SUP35R2E2::KANMX6</i>	SB859, SB884	This study
SY2335	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{SUP35}C leu2-3, 112</i>	SLL6682	This study
SY2336	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{SUP35}C leu2-3, 112 SUP35R1-4</i>	SB775, SLL6682	This study
SY2468	<i>MATa [PSI⁺] ade1-14 his3Δ200::HIS3::P_{SUP35}SUP35R2E1 trp1-289 ura3-52::URA3::P_{SUP35}C leu2-3, 112 SUP35R2E1</i>	SB787, SB883, SLL6682	This study
SY2337	<i>MATa [PSI⁺] ade1-14 his3Δ200::HIS3::P_{Sup35}SUP35R2E2 trp1-289 ura3-52::URA3::P_{SUP35}C leu2-3, 112 sup35::P_{MFA1}SUP35R2E2::KANMX6</i>	SB859, SB884, SLL6682	This study

Materials and Methods

Plasmids

All plasmids are described in table 1. Construction of SB804, SB775, SB776, SB787, SB883, SB859, and SB854 is described in chapter 2.

Copper-inducible NM-GFP plasmids (SB865, SB866, and SB867) were constructed by insertion of a *Bam*HI/*Bse*RI fragment from SB804, SB775, and SB776 respectively into SLL6442 (from S. Lindquist). Repeat number in clones was confirmed by sequencing.

Strains

All strains are described in table 2, and are derivatives of 74-D694. All $[PSI^+]$ strains are $[PSI^+]^{strong}$.

Repeat variant strains (SY2072, SY2057, SY2022, SY2247, and SY2300) were constructed as described in chapter 2. $[PSI^+]$ in SY2057, and SY2022 was cured by overexpression of Hsp104 as previously described (DiSalvo et al., 2011) to generate $[psi^-]$ repeat variant strains (SY2213, and SY2215). $[PSI^+]$ in SY2247 and SY2300 was cured by growth on guanidine HCl to generate $[psi^-]$ repeat variant strains SY2302 and SY2305, respectively. Strains expressing an extra copy of Sup35C (SY2335, SY2336, SY2468 and SY2337) were generated by integration of *Ppu*MI digested SLL6682 in SY2606, SY2057, SY2247, and SY2300, respectively. Transformants were selected by growth on medium

lacking uracil and for red colony color on YPD. $[PSI^+]$ status was confirmed by SDD-AGE.

$[PSI^+]$ Induction

$[PSI^+]$ *de novo* induction was carried out as previously described (Marchante et al., 2013) with the following modifications. SLL2119 was transformed with SB865, SB866, SB867, or SLL6682 and transformants were selected on minimal medium lacking uracil. Strains were grown at 30°C to OD 0.1-0.3 in minimal medium lacking uracil and then grown in the presence or absence of 10 μ M CuSO₄ for 4 hours, after which cultures were plated on YPD for colony color phenotype. For each strain, at least 1000 colonies in at least three independent experiments, with at least three replicates each, were analyzed.

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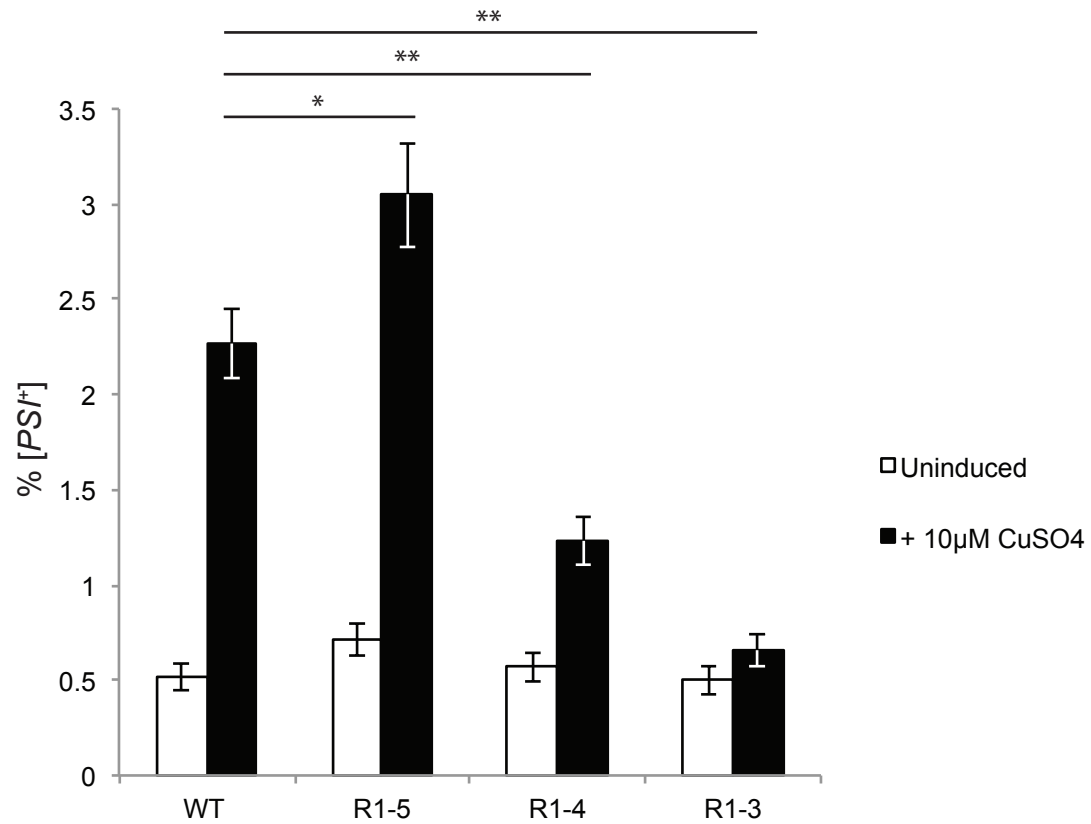


Figure 1. Rates of fragmentation can be linked to prion induction frequency
A $[psi^-]$ strain (SLL2119) was induced to express NM-GFP containing different numbers of repeats with CuSO₄ for 4h. Cells were plated on rich medium and scored by colony color phenotype. Bars represent means, error bars represent standard error. $n \geq 3$, * $p < 0.05$, ** $p < 0.01$, student's t-test.

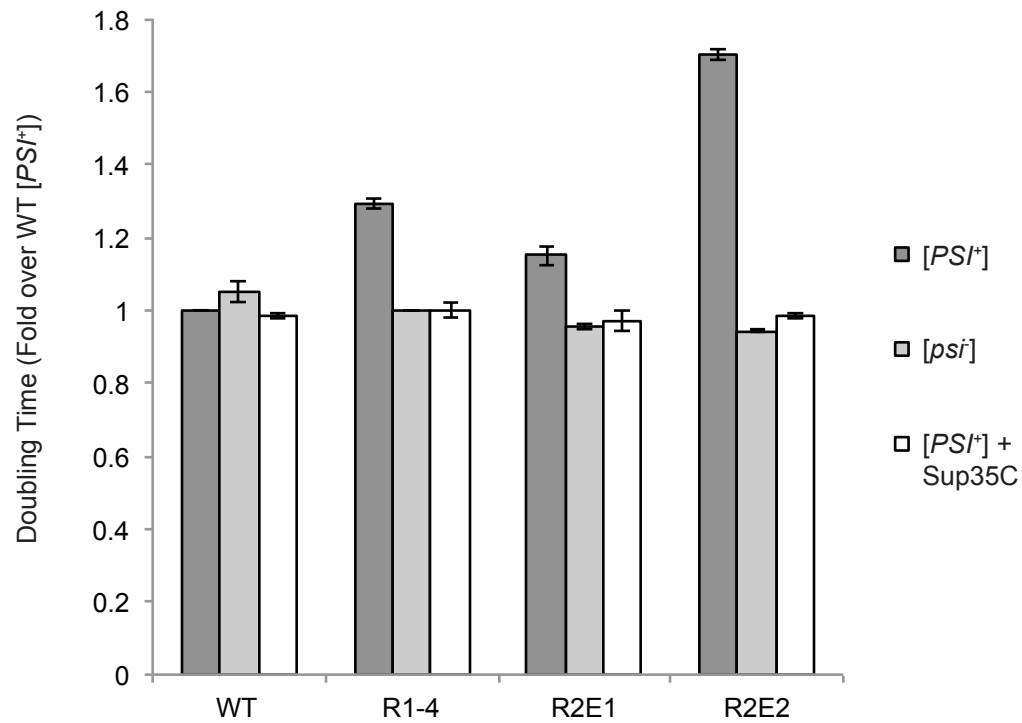


Figure 2. Fragmentation is linked to prion toxicity

The indicated [PSI⁺], [psi⁻], and [PSI⁺] expressing Sup35C strains were grown in rich medium, and doubling times were determined. Bars represent means, error bars represent standard deviation, n=3.

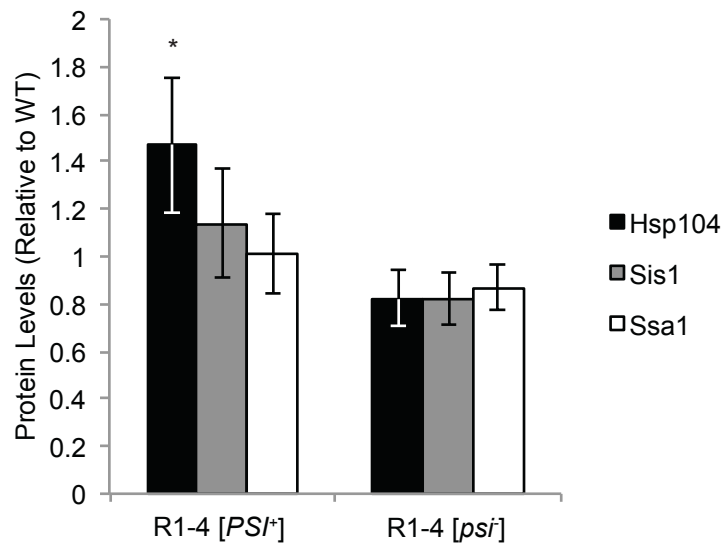


Figure 3. Hsp104 is elevated in R1-4 [*PSI*⁺] strain
R1-4 [*PSI*⁺] (SY2057) and R1-4 [*psi*⁻] (SY2213) strains were analyzed by SDS-PAGE and quantitative immunoblotting for Hsp104 (black), Ssa1 (gray), and Sis1(white). Bars represent means, error bars represent standard deviations. n≥3, *p=10⁻⁵.

Chapter 4:

Conclusion

Prion biogenesis is a multi-step process determined by specific sequence elements

In yeast, prion proteins form self-templating and transmissible aggregates, which confer new phenotypes. In order for these phenotypes to remain stable, the prion aggregates must first recruit non-prion, soluble protein and template its conversion to the prion state. Second, aggregates must be fragmented by molecular chaperones to generate new templates for conversion and to ensure that aggregates remain small enough to be transmitted to daughter cells upon cell division (Sindi and Serio, 2009). In yeast, fragmentation is carried out by the molecular chaperone Hsp104 and its co-chaperones Ssa1 (Hsp70) and Sis1 (Hsp40) (Chernoff et al., 1995; Higurashi et al., 2008; Satpute-Krishnan et al., 2007; Tipton et al., 2008). Hsp104 is a hexameric, barrel-shaped AAA+ ATPase, and is recruited to prion aggregates by Ssa1 and Sis1 (Higurashi et al., 2008; Tipton et al., 2008; Winkler et al., 2012). Following binding to prion aggregates, Hsp104 extracts a Sup35 monomer by threading it through its central pore (Tessarz et al., 2008). The threading and removal of a monomer from the aggregate results in severing of the aggregate, resulting in multiple smaller aggregates (Lum et al., 2004; Tyedmers et al., 2010).

The primary sequence of the yeast prion Sup35 contains an N-terminal Q/N-rich tract, which has been previously shown to drive conversion of soluble protein to the prion state (Kushnirov et al., 1988; Osherovich et al., 2004). Following the Q/N-rich tract, there are five and a half imperfect oligopeptide repeats (Kushnirov

et al., 1988). Although the precise mechanism was unclear, the repeats have been previously implicated in prion propagation (Borchsenius et al., 2001; Osherovich et al., 2004; Parham et al., 2001). Here, we show that the repeats are an important determinant of the efficiency by which prion aggregates are fragmented by molecular chaperones. We propose that the repeats act to stall processing by Hsp104, such that when more repeats are present, Hsp104 must repeatedly engage the substrate to extract a monomer from the aggregate. This repeated action by Hsp104 applies force to the aggregate, leading to its destabilization and increased fragmentation. In contrast, when repeats are deleted, Hsp104 is more easily able to process through the repeat region, leading to monomer extraction without aggregate destabilization. Thus, repeat number is directly correlated with fragmentation rate. These changes to fragmentation observed in our repeat variant strains also lead to differences in other aspects of prion biology, as increasing or decreasing fragmentation by altering repeats number results in toxicity.

In addition, we have identified a new element, the Repeat Proximal Region (RPR), which modulates the effect of the repeats. When the RPR is deleted, more repeats are required to support $[PSI^+]$. We show that the RPR is also an important determinant of fragmentation but does not appear to be acting in the same way as the repeats. We hypothesized that the RPR may serve as a binding site for Ssa1 (Hsp70), as it contains a predicted Hsp70 binding site (Van Durme et al., 2009). In addition to the predicted binding site in the RPR, Sup35 contains several other predicted Hsp70 binding sites. Thus, in our model,

deletion in the RPR would reduce but not abolish Hsp70 binding to Sup35 aggregates. Ssa1 is required to recruit Hsp104 to prion aggregates *in vivo* (Winkler et al., 2012), suggesting that a reduction in Ssa1 binding might result in less Hsp104 recruitment, and thereby less fragmentation. Because the repeat deletions also decrease fragmentation, additional deletion of the RPR in the context of a repeat deletion could push fragmentation below a critical threshold, leading to prion loss as observed in the R1-4 Δ RPR strain (Chapter 2, Figure 1B, 4B). In the future, to determine if the RPR indeed acts as an Ssa1 binding site, we could isolate Sup35 aggregates by immunocapture and quantitatively immunoblot for Ssa1. If deletion of the RPR reduces Ssa1 binding, we would expect less Ssa1 to co-IC with Sup35 in the Δ RPR strain, compared to WT.

Factors that influence recognition/processing by AAA+ ATPases

We propose that the repeats found in the Sup35/[PSI⁺] prion alter the ability of Sup35 to be processed by Hsp104 *in vivo* (see chapter 2). Indeed this mechanism appears to be conserved among other AAA+ ATPase-substrate pairs. While Hsp104 is the central AAA+ ATPase required for fragmentation and propagation of Sup35 prion aggregates (Chernoff et al., 1995), much of the work in understanding how substrate features affect processing was originally done using the bacterial chaperones ClpA and ClpX, which function in conjunction with the ClpP protease, the bacterial proteases FtsH and Lon, and the mammalian proteasome, which all contain members of the AAA+ ATPase family. The

proteases all share structural similarity and are composed of multiple subunits that form a ring structure with a central channel (Horwich et al., 1999). The active sites for proteolysis are buried inside the channels, and the sizes of the channels are such that substrates must first be unfolded to access the active sites (Johnston et al., 1995; Wenzel and Baumeister, 1995). The AAA+ ATPase components function to unfold substrates for these proteases (Horwich et al., 1999), and through studies of these factors, several features of the substrates themselves have been shown to be important for efficient processing, including local protein structure, thermodynamic stability, and sequence complexity (Hoyt et al., 2006; Kenniston et al., 2005; Martin et al., 2008). Furthermore, these features can act together to promote efficient recognition and processing by AAA+ ATPases (Hoyt et al., 2006; Martin et al., 2005; Too et al., 2013). Due to the conserved architecture of AAA+ ATPases, we have used these studies as a model to understand how the Sup35 oligopeptide repeat region might interact with Hsp104.

Local protein structure

Work done by Baker and Sauer has implicated local protein structure as an important factor in the degradation of model substrates by the ClpX-ClpP complex (ClpXP). ClpXP can operate at multiple speeds: a faster speed (~600 ATP/min) when degrading denatured or unstable substrates, and a slower speed (~150 ATP/min) when it encounters folded domains of native substrates

(Kenniston et al., 2003), suggesting an important role for local protein structure in resisting degradation. Substrates with stably folded structures adjacent to the degradation tag were less efficiently degraded, indicating that the protein structure immediately adjacent to the point of initiation is an important determinant for degradation (Kenniston et al., 2003; Martin et al., 2008), possibly because the amount of force required to unfold and translocate a folded domain is proportional to its stability.

In addition to the stability of the local structure, the secondary structure also affects how a protein is processed by these AAA+ proteases. Substrates in which the degradation signal is adjacent to a α helix are more easily degraded than those where the degradation signal is adjacent to a β sheet (Lee et al., 2001). This finding correlates with the fact that β sheets are much more resistant to mechanical unfolding than α helices (Wilcox et al., 2005) and is particularly interesting given that protease-resistant protein aggregates implicated in neurodegenerative disease primarily exist in the β -sheet conformation, whereas their non-aggregated precursors tend to be more enriched for α helices (Lee et al., 2001; Verhoef et al., 2002).

Multi-domain proteins provide an additional obstacle to degradation. In order for complete degradation to occur in these cases, ClpXP must unfold and translocate multiple domains without dissociation of the enzyme. Indeed, after successfully degrading one domain, ClpXP can stall when it encounters a second stably folded domain, resulting in release of a peptide containing a ~37 residue

tail adjacent to the folded domain. The length of this tail is sufficient to span from the entry of the ATPase pore to the first active site of the protease. Importantly, these partially degraded substrates are not re-targeted for degradation (Kenniston et al., 2005) and may have a propensity to form insoluble aggregates (Venkatraman et al., 2004). Indeed, protein aggregation has been shown to impair the function of the ubiquitin proteasome system in eukaryotes. It has been suggested that this inhibition is due to saturation of the proteasomes such that there are not enough free proteasomes to degrade other substrates (Bence et al., 2001). However, later work shows that the proteasome is still able to degrade non-aggregated substrates in cells containing polyQ inclusions (Verhoef et al., 2002), indicating that the inability to degrade these aggregates may be due to the formation of a stable amyloid structure.

The importance of local protein structure is not a unique feature of the ClpXP system. Similar effects are seen with ClpAP, the bacterial Lon protease, as well as both the yeast and mammalian proteasome. In all of these cases, partially degraded substrates are released when the AAA+ machinery encounters a tightly folded domain, and the released polypeptide is resistant to degradation (Lee et al., 2001; Orian et al., 1999; Tian et al., 2005; Wohlever et al., 2014).

Interestingly the importance of local structure has also been observed for multi-protein complexes, including the transcription factors NF- κ B in mammals, and Spt23p and Mga2p in yeast. These proteins exist in a full length inactive form, which is partially degraded by the proteasome to yield an active, truncated

form. Efficient processing and release of the active form is dependent on homodimerization, suggesting that the active domains are left intact because they are tightly associated with other molecules and the proteasome is unable to degrade them (Rape and Jentsch, 2002).

Thermodynamic stability

In addition to local structure, global thermodynamic stability can also influence the efficiency with which substrates are unfolded and degraded by AAA+ proteases. Because the proteases act by unraveling a substrate from one end starting from the degradation signal, these powerful machines are able to unfold even very thermodynamically stable substrates by changing the unfolding pathway of the substrate (Lee et al., 2001). However, unfolding of more stable substrates is not always energetically favorable as total ATP consumption increases with substrate stability (Kenniston et al., 2003). In addition when multiple substrates with different stabilities are in competition with each other, ClpXP preferentially degrades the less stable substrates. This difference in degradation can be explained by the fact that for more stable substrates, ClpXP binds and dissociates multiple times before it commits to degradation. In contrast, unstable substrates can be immediately unfolded without repeated cycles of binding and ATP hydrolysis (Kenniston et al., 2005). Indeed, at low levels of ATP, ClpXP loses the ability to degrade stably folded substrates (Martin et al., 2008).

While global thermodynamic stability of the substrate influences degradation by all the AAA+ proteases discussed here, some are more sensitive to changes in thermodynamic stability than others. For example, ClpXP and ClpAP have strong unfoldases and can thus unfold very stable substrates. This is also true of the eukaryotic proteasome, where native regulatory proteins may need to be quickly degraded (Koodathingal et al., 2009). In contrast, the FtsH protease has a very weak unfoldase activity and therefore cannot degrade stably folded proteins (Herman et al., 2003).

Degradation of substrates by FtsH is inversely related to their thermodynamic stability. As a heat shock protein, FtsH's main role is to translocate and degrade unfolded proteins (Herman et al., 2003). FtsH degrades membrane proteins, and its main criteria for recognition is that its substrates contain a ~20 amino acid tail (Chiba et al., 2000). With such a general criterion for substrate recognition, it has been proposed that FtsH uses the thermodynamic stability of its substrates to discriminate among targets after the binding step has occurred. Indeed, FtsH can bind to but not degrade the model substrates DHFR and barnase, which have tightly folded structures (Herman et al., 2003; Koodathingal et al., 2009). However, when mutations that result in a lower global thermodynamic stability without altering the overall structure are introduced into these proteins, FtsH then efficiently degrades them. In addition, FtsH substrates are degraded faster at elevated temperatures, and this difference is not due to a change in enzyme activity, indicating that a change in

the substrate, likely its unfolding at increased temperature, accounts for this increase in activity (Herman et al., 2003).

Sequence complexity

Sequence identity is important for the recognition and binding step of proteolysis because the proteases tend to recognize specific degradation tags or modified residues. Similarly, mass, shape, and flexibility of residues are all positively correlated with the ability of the AAA+ proteases to translocate substrates (Too et al., 2013). However, precise sequence identity does not seem to be an essential determinant in substrate processing. Instead, sequence complexity seems to be the determining factor, with regions of lower complexity being less efficiently translocated through the central channel. AAA+ proteases use aromatic paddles to move the substrate through the degradation channel (Lum et al., 2004; Schlieker et al., 2004; Weibezahn et al., 2004). It is likely that regions of lower complexity have fewer features that the paddles can interact with, and therefore these regions cannot efficiently be translocated (Kraut, 2013). Indeed, it has been suggested that substrate sequence is important for events that take place in between enzyme power strokes, rather than during power strokes (Too et al., 2013), such as the ability of substrates to refold following partial unfolding.

Additionally, when a region of low sequence complexity is followed by a stably folded domain, AAA+ proteases are unable to complete degradation, and it has been suggested that the combination of these features functions as a stop-transfer signal (Orian et al., 1999; Rape and Jentsch, 2002). Degradation was stalled when a glycine-rich, serine-rich, or asparagine-rich region was proximal to a stably folded domain, indicating that the precise identity of the amino acids is not important, but rather that the proteases are unable to translocate regions of low complexity (Koodathingal et al., 2009). In one model, the protease does not efficiently interact with these regions, resulting in less forward movement of the substrate with each round of ATP hydrolysis. This translates into less force being applied to the folded domain, leading to less unfolding, thereby increasing the likelihood that domain will snap back to a folded structure before it can enter the central pore (Kraut, 2013). Consistent with this idea, unfolding and degradation of GFP by ClpXP requires rapid ATP hydrolysis because the rate of translocation must exceed the rate of substrate refolding. More specifically, if refolding of the substrate is sufficiently fast, the substrate will not be degraded due to a failure to be translocated. In contrast, if substrate refolding is slow, enough of the unfolded polypeptide can be pulled into the channel to prevent refolding (Martin et al., 2008). Thus, the relative rates of substrate refolding and translocation (via force applied on the substrate by the AAA+ machine) are an important determinant of the ability of substrates to be degraded.

Low sequence complexity has been implicated in the failure to efficiently degrade several substrates, including polyQ stretches (Kraut, 2013), the Epstein

Barr Nuclear Antigen 1 (EBNA1) (Hoyt et al., 2006), and the transcription factors NF- κ B and *Cubitus interruptus* (Ci) (Figure 1) (Lin and Ghosh, 1996; Orian et al., 1999; Tian et al., 2005). Importantly, lack of protease processing may be occurring by a similar mechanism in all of these cases.

Processing of polyQ proteins by the proteasome. PolyQ substrates decrease processivity of the proteasome in a length-dependent manner when 11, 33 or 53 residues are fused to a stably folded DHFR domain. In addition, while the presence of 33 residues slowed down processing, only 53 residues completely prevented the degradation of DHFR (Kraut, 2013). This finding is consistent with the fact that at least 35 polyQ residues are required for disease progression in the case of Huntington's Disease and the spinocerebellar ataxias (Williams and Paulson, 2008).

The eukaryotic proteasome is unable to degrade polyQ fragments. Instead, these fragments are released whole and left for other proteases to degrade and clear from the cell (Venkatraman et al., 2004). However, these partially degraded fragments are prone to aggregation and can serve as a nucleus to drive other polyQ proteins into cellular inclusions (Navon and Goldberg, 2001; Venkatraman et al., 2004). Once aggregated, these proteins are additionally resistant to degradation and unable to be cleared from cells (Verhoef et al., 2002).

Processing of EBNA1 by the proteasome. The EBNA1 protein of the Epstein-Barr virus is resistant to proteosomal degradation and contains an amino

acid tract between 60 and 300 residues long, consisting entirely of glycine and alanine residues (glycine/alanine repeat, GAr). The GAr acts in *cis* to prevent proteosomal degradation of EBNA1 and is sufficient to prevent degradation, in a length-dependent manner when transferred to other, unrelated proteins (Levitskaya et al., 1997; Too et al., 2013).

The GAr does not prevent recognition by the proteasome but rather causes the proteosomal machinery to stall and release the partially degraded substrate (Zhang and Coffino, 2004). In addition, stalling depends on the presence of a stable, folded domain near the GAr, supporting a model where the GAr is processed inefficiently by the proteasome, reducing the amount of energy that can be transferred to unfolding the substrate, resulting in a failure to unfold the substrate (Hoyt et al., 2006). Several lines of evidence support this model. First, mutations that destabilize the β sheet structures adjacent to the GAr reduce its ability to impede degradation. Second, the spacing between the GAr and the folded domain is an important factor. If the GAr is too far away from the folded domain, stalling is reduced, suggesting that the folded domain must arrive at the entrance to the pore at the same time that the GAr reaches the active site (Hoyt et al., 2006). Notably, replacement of the GAr with a glycine-only or alanine-only stretch results in a decrease in inhibition, suggesting that the presence of small side chains with few features cannot be the only relevant property (Too et al., 2013).

Processing of NF- κ B by the proteasome. The NF- κ B transcription factor contains two subunits: p50 and p65 (Baeuerle and Baltimore, 1989; Ghosh and Baltimore, 1990). The p50 subunit is generated from an inactive precursor protein, p105, and this generation is dependent on the proteasome, which rapidly degrades the C-terminal portion of p105 while leaving the N-terminal portion intact (Ghosh and Baltimore, 1990; Palombella et al., 1994). NF- κ B subunit p50 contains a 35 amino acid, glycine-rich region (GRR), which is essential for processing of p105 and generation of p50 (Lin and Ghosh, 1996). In addition, the GRR acts to stabilize p50 after its generation, and it is likely that both the formation and stabilization of p50 by the GRR occur through the same mechanism (Orian et al., 1999). Interestingly the GRR contains two glycine-alanine repeats, and similarly to EBNA1, when these alanines are mutated to glycine, generation of the p50 fragment is significantly reduced. However, replacement of the alanines with valines only causes a slight decrease in processing, indicating an importance for residues with a side chain rather than a requirement for a specific residue (Orian et al., 1999). Indeed, when the GRR is replaced with either a serine-rich region or an asparagine-rich region, the formation of the partially degraded product is not reduced (Tian et al., 2005). There are conflicting reports on whether the GRR can serve as a transferable sequence and cause processing defects when fused to other proteins. When the GRR was fused in between two unrelated proteins (gp10 and GST), it could still act as a processing signal (Lin and Ghosh, 1996). However, in a subsequent study where the GRR was fused in between p53 and ODC, no processing defect

was observed (Orian et al., 1999), indicating that the GRR is not sufficient to stall processing on its own but rather requires some other feature of the substrate for efficient stalling.

Indeed, the presence of a tightly folded domain proximal to the GRR is required for processing to occur (Tian et al., 2005). For NF- κ B, the folded domain is the Rel homology domain, which is the site of homodimerization for p105, and when the GRR is placed next to a less thermodynamically stable domain, p50 is no longer accumulated (Tian et al., 2005). In addition, when mutations are made in NF- κ B that destabilize the hydrophobic core without altering targeting of p105 to the proteasome, p50 accumulation decreases (Lee et al., 2001). To exert its effects, the GRR must be located between the degradation signal and the folded domain. In addition, similarly to what has been observed for EBNA1 and polyQ, the spacing between the GRR and the folded domain is important. Formation of p50 is abolished when the GRR is moved more than 35 residues away from the dimerization domain (Tian et al., 2005). Thus, the presence of the Rel dimerization domain followed by the GRR serves as a stop-processing signal. By inhibiting further translocation of the polypeptide into the channel, the active form of p50 is released from the proteasome (Hoyt et al., 2006; Kraut, 2013).

Processing of Cubitus interruptus (Ci) by the proteasome. Similar to NF- κ B, the *Drosophila* transcription factor Ci exists in two forms: a full-length, inactive form (Ci-155), which is processed by the proteasome to yield the

truncated, active form (Ci-75) (Cohen, 2003). Also reminiscent of EBNA1 and NF- κ B, Ci contains a tightly folded domain consisting of five zinc fingers, followed by three stretches of simple sequence: an asparagine/glutamine-rich stretch, a serine-rich stretch, and an aspartate-rich stretch. Destabilization or deletion of the Zn-finger domain or deletion of the simple sequences results in a loss of active, processed Ci, both *in vivo* and *in vitro* (Tian et al., 2005; Wang and Price, 2008). Furthermore, replacement of the Zn-finger domain with another tightly folded domain restores processing, and the amount of active Ci produced is proportional to the stability of the domain, indicating that the Zn-finger domains are not required for dimerization, as is the case for NF- κ B but rather are important because of their tightly folded tertiary structure (Wang and Price, 2008). In addition, replacement of the simple sequences in Ci with the GRR from NF- κ B or another serine-rich sequence also restores fragment formation, again indicating that sequence complexity and not sequence identity is the primary determinant for processing (Tian et al., 2005).

Evidence for a conserved mechanism

For all of the proteins discussed above, the combination of simple sequence followed by a folded domain in the direction of proteasome movement leads to partial processing of the substrate. These features have been proposed to act as stop-transfer sequences, where the proteasome is not able to efficiently interact with the substrate and thus is unable to transfer the energy required to unfold stable domains, ultimately resulting in the release of a partially degraded

product from the protease (Hoyt et al., 2006). Importantly, these effects are not limited to the proteasome but have also been observed in studies using ClpXP, suggesting that these feature exert their affects on AAA+ ATPases through a universal mechanism (Too et al., 2013).

It is also important to note that the effects of the features discussed above are not limited to AAA+ protease systems. Similar effects have been seen in studies with other protein translocases, specifically those proteins involved in mitochondrial import. Similarly to the AAA+-associated protease substrates, proteins must be completely unfolded in order to be translocated into the mitochondria, and translocation proceeds in a sequential manner (Eilers and Schatz; Schwartz et al., 1999). The ability of substrates to be imported is affected by the local structure immediately adjacent to the import tag, and similarly to ClpXP substrates, α helixes are more susceptible than β sheets to import when located adjacent to the import start site (Lee et al., 2001; Wilcox et al., 2005). Additionally, the ability of a protein to be imported is inversely correlated with its resistance to mechanical unfolding but does not correlate well with the global thermodynamic stability of the protein, indicating that the force required for unfolding does not have to resonate through the entire protein structure but rather is concentrated at the first stably folded structure (Wilcox et al., 2005).

Processing of Sup35 by Hsp104

Interestingly, Sup35 aggregates contain features similar to those proteins that are not efficiently processed by AAA+ proteases. We propose that the oligopeptide repeat region serves as the simple sequence, while the Q/N-rich region serves as the tightly folded domain. Indeed, like other hard to process substrates, the Q/N-rich region (amino acids 1-41) of the Sup35 protein in prion aggregates is composed primarily of β -sheets, and the tightness of this aggregation has been demonstrated by H/D exchange, where little exchange occurs within the first thirty seven amino acids (Toyama et al., 2007). Additionally, mutations that decrease the thermodynamic stability of prion aggregates result in increased fragmentation by Hsp104, similar to the effects observed in the ClpXP system (DiSalvo et al., 2011; Kenniston et al., 2005; Martin et al., 2008). The oligopeptide repeats (amino acids 42-97) immediately follow the Q/N rich region, and the spacing of these elements is consistent with studies on ClpXP, where substrates are released when the enzyme encounters a tightly folded domain, and the released product contains the domain plus an amino acid tail, which is sufficient to span from the active site to the pore entrance of the enzyme (Kenniston et al., 2005). Thus, the spacing between the repeat region and the aggregation domain is optimal to promote Hsp104 stalling, and it is the combination of the “slippery” nature of the repeats and its proximity to the aggregation domain, which requires more energy to unfold, that leads to inefficient threading of Sup35 by Hsp104 when it encounters the repeat region.

Thus, we believe that the repeats act to stall processing, similarly to what has been previously demonstrated for the glycine-alanine repeats in EBNA-1 and

the glycine-rich region of NF-KB (Hoyt et al., 2006; Lin and Ghosh, 1996; Orian et al., 1999). If this model is correct, we might expect the GAR or GRR to functionally replace the repeats in Sup35. In order to test this hypothesis in the future, we would construct strains containing the appropriate replacements, and monitor their ability to support the prion state by phenotype, aggregate size, and aggregate properties. In addition, to further support our model that the repeat variants influence the ability of Sup35 to be threaded by Hsp104, we could use an Hsp104 variant that couples to the bacterial protease, ClpP (Tipton et al., 2008; Weibezahn et al., 2004). In this system, following threading through Hsp104, substrates are fed into the central pore of ClpP, leading to their degradation (Tessarz et al., 2008; Weibezahn et al., 2004). Using this system in the context of our luciferase reporter constructs (see Chapter 2), if the presence of different numbers of repeats affects threading, we might expect to see differential release of proteolytic fragments, similarly to what has been previously observed in studies with ClpXP (Kenniston et al., 2003; Koodathingal et al., 2009), the Lon protease (Wohlever et al., 2014), and the mammalian proteasome (Lin and Ghosh, 1996). Specifically, if our model that increasing repeat number promotes enzyme stalling is correct, we would expect repeat number to directly correlate with the amount of proteolytic fragments released.

In addition, prion aggregates do not exist in a single conformation but rather can exist in a variety of conformations, termed prion strains, and these conformations can give rise to a spectrum of phenotypes (Legname et al., 2006; Tanaka et al., 2006). The work described here was performed using the

$[PSI^+]^{\text{strong}}$ prion strain. The role of repeats has not been explored in other prion strains, and one remaining question is whether the repeats fulfill the same function for all conformational variants. Another commonly used laboratory prion strain is $[PSI^+]^{\text{weak}}$, which has less stop codon read-through activity compared to $[PSI^+]^{\text{strong}}$, resulting in a pink phenotype and very little growth on medium lacking adenine (Kryndushkin et al., 2003). This phenotypic difference can partially be explained by the fact that compared to $[PSI^+]^{\text{strong}}$, $[PSI^+]^{\text{weak}}$ contains a larger amyloid core (amino acids 1-37 in $[PSI^+]^{\text{strong}}$, compared to 1-70 in $[PSI^+]^{\text{weak}}$), which extends into the third repeat (Toyama et al., 2007). Because spacing between the tightly folded domain and the region of simple sequence is an important determinant for AAA+ ATPase stalling (Kenniston et al., 2005; Orian et al., 1999), one might expect that the repeats may not behave similarly when in different prion conformations. Indeed, preliminary data suggest that at least five repeats are required to support $[PSI^+]^{\text{weak}}$, compared with four repeats in $[PSI^+]^{\text{strong}}$, consistent with the idea that the repeats must span a minimum distance from the amyloid core in order to support $[PSI^+]$. To test whether the repeats act in a similar way in different prion strains, we could introduce $[PSI^+]^{\text{weak}}$ into our repeat variant collection and monitor prion dynamics by phenotype, aggregate size, and aggregate properties.

In addition, $[PSI^+]^{\text{strong}}$ is particularly sensitive to a mutation in the Gly-Gly motif of the second repeat (G58D), but is not as sensitive to mutations in the Gly-Gly motif of other repeats (Marchante et al., 2013). We speculate this specificity might arise because G58/59 is uniquely positioned with respect to the amyloid

core, such that G58/59 reaches Hsp104's aromatic paddles at the same time that the amyloid core reaches the pore entrance. Thus, any increase in substrate architecture at G58/59 would create increased structural elements for Hsp104 to interact with at the same time that more energy is required to unfold the aggregation domain, thereby limiting stalling. Consistent with this idea, Hsp104 binding to Sup35, as determined by peptide array, occurs primarily in the Sup35M/C regions (Lum et al., 2008), suggesting that Hsp104 processing of Sup35 initiates C terminal to the prion forming domain. Interestingly $[PSI^+]^{\text{weak}}$ is less sensitive to the G58D mutation (Derkatch et al., 1999), possibly because in this case the G58/59 residues are in the amyloid core and thus not positioned in a way that would increase stalling. If our model is correct, we might expect $[PSI^+]^{\text{weak}}$ to be more sensitive to a mutation in the Gly-Gly motif of repeat 5 (G86/87) or repeat 6 (G96/97), as these residues are located a sufficient distance from the amyloid core to promote stalling (Kenniston et al., 2005; Kushnirov et al., 1988; Toyama et al., 2007)

Implications for preservation of the prion state

Fragmentation efficiency is critically important for maintenance of the prion state, as either increases or decreases in fragmentation can result in prion loss. For example, increasing fragmentation rates by either up-regulation of chaperones following heat stress or incorporation of an aggregate destabilizing mutant results in prion loss (DiSalvo et al., 2011; Klaips et al., 2014).

Conversely, decreasing fragmentation by inhibition of Hsp104 or reducing the ratio of Hsp104:Sup35 also results in increased prion loss (DiSalvo et al., 2011; Eaglestone et al., 2000; Ness et al., 2002; Winkler et al., 2012). Similarly, we show that decreasing fragmentation by decreasing repeat number results in prion loss. While we did not observe an increase in prion loss as fragmentation increased with increasing repeat number, in this case increased fragmentation resulted in cellular toxicity (Chapter 3), and it remains possible that prion loss may be observed if the repeat number is expanded beyond 7.5 repeats. The narrow range of repeat numbers able to support $[PSI^+]$ suggests that precise levels of fragmentation are important for maintaining both the prion and non-prion states while avoiding toxicity.

Consistent with this interpretation, many prion and amyloid proteins contain similar numbers of oligopeptide repeats. For example, all yeast Sup35 homologs that are capable of forming a prion contain between 5 and 6 oligopeptide repeats (Nakayashiki et al., 2001), mammalian PrP contains 5 copies of an octarepeat (Kretzschmar et al., 1986), and functional amyloids CsgA in *E. coli* and Pmel17 in humans contain 5 and 10 copies of an oligopeptide repeat, respectively (Cherny et al., 2005; Kwon et al., 1991). In all cases, repeats are enriched for glutamine, glycine, and proline (Cherny et al., 2005; Kretzschmar et al., 1986; Kwon et al., 1991; Nakayashiki et al., 2001), and deletion of the repeat region abrogates the ability of the protein to adopt the amyloid conformation (Cherny et al., 2005; Nichols et al., 2003; Osherovich et al., 2004; Yamaguchi et al., 2012). Conversely, expansions of the repeat region of

PrP or Sup35 promote amyloid formation *in vitro* and *in vivo* (Chiesa et al., 2000; Hess et al., 2007; Jansen et al., 2009; Leliveld et al., 2006; Liu and Lindquist, 1999). These similarities observed across diverse organisms and among proteins with diverse functions suggest that the repeats may act in a similar way to promote the propagation of both the amyloid and non-amyloid states. Indeed, it has been suggested that the amyloid form could be either advantageous or disadvantageous to the organism depending on the environmental conditions, thereby making the ability to switch between states critically important for the organism's survival (Halfmann et al., 2010; True and Lindquist, 2000). Many yeast prions are spontaneously gained or lost at a frequency of $\sim 10^{-7}$ (Lancaster et al., 2010; Liu and Lindquist, 1999), suggesting that a subset of any population of wild yeast will have gained the prion state. Thus, if environmental conditions change such that the prion state becomes advantageous, those cells have a better chance to survive. Additionally, because prion loss occurs at a similar rate, if the environment were to fluctuate so that the prion state was no longer advantageous, there would still be a subset of the population that was able to survive (Halfmann et al., 2010). Thus, the ability to switch between prion and non-prion states promotes population survival through a variety of environmental fluctuations. Indeed, yeast prions are enriched for transcriptional and translational regulators (Alberti et al., 2009), supporting the idea that transition to the prion state can act as a switch to alter cellular pathways leading to broad phenotypic effects.

Amyloid Processing and Cellular Toxicity

Fragmentation is a critical step in prion biogenesis, as it both creates new templates for conversion and ensures that aggregates remain small enough to be inherited by daughter cells. Maintaining a precise level of fragmentation is critically important for maintaining both [PRION+] and [prion-] states (Figure 2). Indeed, altering fragmentation levels results in alteration of aggregate properties, including steady-state aggregate size, aggregate number, and efficiency of incorporation of soluble protein (see Chapter 2), which can result in cellular toxicity (see Chapter 3). Similarly, lack of processing is associated with amyloid toxicity through the sequestration of cellular co-factors and impairment of cellular quality control machinery.

Toxicity induced by sequestration of essential cellular factors

In amyloid diseases, such as Huntington's, Alzheimer's and Parkinson's disease, protein aggregation results in a toxic gain of function. One hypothesis to explain this toxicity is that when protein oligomers form, they provide an increase in exposed hydrophobic surfaces that can then promote aberrant interactions with other proteins, including essential cellular factors (Bolognesi et al., 2010; Gidalevitz et al., 2006; Olzscha et al., 2011). Consistent with this hypothesis, cytotoxicity of amyloid aggregates correlates with their ability to sequester cellular proteins. Furthermore, the sequestered proteins are enriched for those involved in more protein-protein interactions, suggesting that they occupy central positions in cellular networks (Olzscha et al., 2011). In addition,

proteins that interact with amyloid aggregates are more frequently linked with proteins implicated in neurodegenerative disease than the average cellular protein (Olzscha et al., 2011; Raychaudhuri et al., 2009). Interestingly, protein interactors largely belonged to one of two groups: those enriched for intrinsically unstructured regions (IURs) and large, multi-domain proteins. The former mostly contains already synthesized proteins that are prone to aggregation even in their folded state, while the latter contains proteins that are prone to aggregate during and immediately after synthesis (Olzscha et al., 2011). This enrichment for IURs suggests that amyloid aggregates may be acting in a chaperone-like way by shielding exposed hydrophobic surfaces, but they cannot promote refolding, leading to enhanced sequestration of essential cellular factors.

Prion aggregates, which are not toxic under normal expression conditions, result in cytotoxicity upon their overexpression through a similar sequestration mechanism (Holmes et al. 2014; Vishveshwara et al., 2009). For example, $[PSI^+]$ prion aggregates can become toxic upon overexpression of either the Sup35 prion domain (PrD) or full-length Sup35. Toxicity induced by overexpression of Sup35 PrD is likely due to the sequestration of soluble, functional Sup35, as well as a loss of smaller, functionally engaged aggregates, and expression of a single copy of the functional domain (Sup35C), which cannot incorporate into prion aggregates, suppresses this toxicity (Pezza et al., 2014; Vishveshwara et al., 2009). Toxicity induced by overexpression of full-length Sup35, however, cannot be rescued by expression of a single copy of Sup35C. In this case, rescue requires similar overexpression of Sup35C or Sup45 (Derkatch et al., 1998;

Vishveshwara et al., 2009). Sup45 binding to Sup35 is mediated by the C-terminal region of Sup35 and is required for efficient translation termination (Ito et al., 1998; Ter-Avanesyan et al., 1994). Thus, overexpression of full-length Sup35 likely sequesters Sup45 into larger, non-functional aggregates. Co-overexpression of Sup35C or Sup45 will then titrate Sup45 or Sup35, respectively away from aggregates to restore efficient termination (Vishveshwara et al., 2009). While it remains possible that $[PSI^+]$ aggregates may sequester additional cellular cofactors, a genome-wide overexpression screen only identified Sup45 and Hsp104, which cures $[PSI^+]$ (Chernoff et al., 1995), as factors that are able to rescue Sup35 overexpression-mediated toxicity (Tank and True, 2009). Thus, the toxic effects are likely due to impaired termination due to loss of Sup35 or Sup45 and not to impairment of other cellular pathways.

Overexpression-induced toxicity of prion proteins is not limited to Sup35, as overexpression of Rnq1, the determinant of the $[PIN^+]$ prion, also leads to cytotoxicity in a $[PIN^+]$ dependent manner (Douglas et al., 2008). Rnq1 is a protein of unknown function, and like $[PSI^+]$, at normal expression levels, $[PIN^+]$ is not toxic (Derkatch et al., 2001). Additionally, $[PIN^+]$ is also commonly found in wild yeast strains (Nakayashiki et al., 2005), suggesting that it may even be beneficial in some environments. However, even modest overexpression of the Rnq1 protein leads to toxicity in $[PIN^+]$ cells, and this toxicity can be suppressed by overexpression of the Hsp40 Sis1, which binds to Rnq1 aggregates (Douglas et al., 2008; Lopez et al., 2003). However, sequestration of Sis1 does not account for the observed toxicity because expression of a Sis1 mutant which can

functionally replace WT Sis1 but does not bind Rnq1 aggregates does not rescue toxicity (Douglas et al., 2008). Instead, toxicity has been linked to sequestration of Spc42, an essential component of the spindle pole body, and overexpression of Spc42 also rescues toxicity (Treusch and Lindquist, 2012). Because Sis1 binds Rnq1 aggregates under normal expression levels (Lopez et al., 2003), excess Sis1 may rescue overexpression-induced toxicity by shielding a possible interaction surface from Spc42 (Holmes et al., 2014). Thus, toxicity of prion aggregates as demonstrated by $[PSI^+]$ and $[PIN^+]$ appear to be due to highly specific sequestration of cellular co-factors, rather than inducing general protein misfolding and aggregation, as observed for amyloid aggregates in mammalian cells (Olzscha et al., 2011; Treusch and Lindquist, 2012; Vishveshwara et al., 2009).

Toxicity induced by impairment of cellular quality control pathways

An alternate, but not mutually exclusive, explanation for amyloid-induced toxicity is that amyloid aggregation results in a significant increase in misfolded proteins, which then engage the chaperone machinery, interfering with the normal quality control pathways in the cell and leading to global protein folding defects (Bence et al., 2001; Gidalevitz et al., 2006). Indeed, amyloid aggregates, even in their more toxic forms, fail to induce a stress response (Olzscha et al., 2011; Tank and True, 2009; Tatzelt et al., 1995) and may even sequester essential components of the quality control machinery, including components of the chaperone and proteasome networks (Bence et al., 2001; Cummings et al.,

1998; Olzscha et al., 2011; Verhoef et al., 2002). In addition, cells containing huntingtin aggregates have higher levels of general ubiquitination compared to cells with soluble huntingtin, suggesting an impairment of the ubiquitin-proteasome system (Bence et al., 2001). In one model, impairment of the UPS is a result of the 26S proteasome becoming “clogged” by polyQ substrates and thus being unavailable to fulfill its normal functions in cellular quality control (Bence et al., 2001). However, other studies suggest that the polyQ aggregates induce proteostasis collapse by promoting general protein misfolding (Hipp et al., 2012; Verhoef et al., 2002). In this model, the Hsp70/40 chaperone networks become overburdened as a result of trying to maintain polyQ solubility, thus making them unavailable for their normal functions. Therefore, proteins that normally depend on the Hsp70/40 chaperones for proper folding will be unable to fold and are targeted to the UPS for degradation. Furthermore, once polyQ aggregates form in the cell, they are recalcitrant to degradation (Hipp et al., 2012; Venkatraman et al., 2004; Verhoef et al., 2002), leading to further build up of aggregation-prone proteins. Thus, as a result of trying to prevent polyQ aggregation, the cellular quality control systems become overburdened, leading to cytotoxicity. Consistent with this idea, expression of polyQ-expanded proteins results in loss of function of metastable proteins, and once misfolded, these proteins promote further polyQ aggregation and toxicity (Gidalevitz et al., 2006), suggesting that the cellular environment is extremely sensitive to disruptions in protein folding homeostasis.

As amyloid aggregates accumulate, they initiate a “snowball” effect, promoting the misfolding and aggregation of a variety of cellular proteins. Consistent with this idea, overexpression of chaperones can suppress amyloid aggregation and thereby reverse toxicity. For example, overexpression of Hsp70 reduces the toxicity of A β , one of the major components implicated in Alzheimer’s disease (Magrané et al., 2004), and reduces the accumulation of α -synuclein, which is implicated in Parkinson’s disease, in detergent insoluble inclusions (Klucken et al., 2004). Similarly, in yeast, Hsp70/40 overexpression reduces the formation of large detergent insoluble aggregates and instead promotes the appearance of smaller, detergent soluble inclusions (Muchowski et al., 2000), which may be more easily disposed of by the cellular degradation machinery. Thus, overexpression of the Hsp70/40 chaperone system is able to reduce the aggregation of a variety of amyloidogenic proteins, relieving the cellular quality control machinery and resulting in reduced cytotoxicity. In addition, binding of Hsp70/40 chaperones to amyloid aggregates may act to shield hydrophobic regions, thereby preventing the recruitment of other aggregation-prone proteins, as has been observed for the Rnq1 prion (Treusch and Lindquist, 2012).

Impairing the balance between prion aggregate accumulation and the fragmentation machinery results in toxicity

Together, the examples discussed above suggest that while some amyloids may be beneficial, there must exist a precise balance between their accumulation and the quality control machinery in place to ensure their

propagation. Chaperones are required for the appearance of the amyloid form; however, a failure of amyloids to induce a stress response may actually be one of the determinants of toxicity. Indeed, increased processing of amyloids by chaperones may promote remodeling of protein-protein interactions within aggregates to relieve the sequestration of essential cellular factors.

Consistent with these ideas, we observed [*PSI*⁺]-induced cellular toxicity as a result of either increasing or decreasing fragmentation by changing repeat number (see Chapter 3). When fragmentation is decreased as in R1-4 [*PSI*⁺] cells, the reduced aggregate processing results in toxicity. Our preliminary studies indicate that expression of an extra copy of either Sup45 or Sup35C, which binds Sup45 but cannot join prion aggregates (Derkatch et al., 1998; Ter-Avanesyan et al., 1994; Vishveshwara et al., 2009), rescues toxicity (see Chapter 3), suggesting that sequestration of Sup45 mediates toxicity. Intriguingly, R1-4 does not accumulate to higher levels under these conditions, and the interaction with Sup45 is restricted to the catalytic domain, suggesting that changes in the processing of these complexes impacts the sequestration of Sup45. To test the role of Sup45 in R1-4 toxicity in the future, we could determine if Sup45 localization is different in the R1-4 and WT strains using a Sup45GFP construct. Sup45GFP appears diffuse in WT [*psi*⁻] and [*PSI*⁺] strains (Derkatch et al., 1998), and if R1-4 aggregates are able to sequester more Sup45 than WT aggregates, we would expect Sup45GFP fluorescence to appear punctate in the R1-4 strain, similarly to the Sup45GFP fluorescence pattern observed following overexpression of Sup35NM (Vishveshwara et al., 2009). If the sequestration of

Sup45 is indeed due to a lack of processing, then increasing prion aggregate fragmentation by increasing chaperone levels should rescue toxicity. Interestingly, the R1-4 [*PSI*⁺] strain has slightly elevated Hsp104 but not Ssa1 or Sis1, perhaps in an attempt to restore aggregate fragmentation. However, overexpression of Hsp104 on its own actually inhibits prion fragmentation by promoting non-productive interactions (Winkler et al., 2012). Thus, the elevated levels of Hsp104 may actually accentuate the fragmentation defect of the R1-4 [*PSI*⁺] strain. To test if increasing fragmentation levels rescues toxicity in the R1-4 strain, growth rates should be compared in conditions that increase fragmentation by promoting global increases in chaperone levels such as heat shock (Klaips et al., 2014). In addition, if increasing fragmentation restores termination by releasing Sup45, we would expect the Sup45GFP fluorescence pattern in heat shocked R1-4 cells to more closely resemble WT.

Shifting the balance between fragmentation and prion aggregates too far in the opposite direction also results in cellular toxicity, as we observed with the repeat expansions. In this case, increased fragmentation results in an increase in the number of templates available for conversion, and drives more soluble Sup35 into prion aggregates. Thus, there is not sufficient functional Sup35 available for efficient termination to occur. If the toxicity of the repeat expansion strains is due solely to the increased recruitment of soluble Sup35 into prion aggregates, then mutations that decrease conversion, such as the dominant-negative mutant Q24R (DiSalvo et al., 2011), should at least partially rescue the growth defect. However, it is also possible that the increased number of

templates results in toxicity by promoting misfolding and aggregation of other cellular factors, such as Sup45. If this is the case, we would expect over-expression of Sup45 to rescue the growth defect in these strains. While other essential cellular factors are not known to be sequestered by Sup35 (Tank and True, 2009; Vishveshwara et al., 2009), another possibility is that the repeat expansion creates a unique interaction surface that promotes an interaction not seen in WT Sup35 aggregates. To determine if this is the case, Sup35 aggregates could be isolated under non-denaturing conditions and interacting proteins could be identified by SDS-PAGE and mass spectrometry in the future.

Conclusion

Together our work presented here highlights the importance of the interplay between propagation of prion aggregates and the cellular quality control pathways that promote their inheritance. In order for protein-based phenotypes to persist, there must exist a precise balance between protein accumulation, protein aggregation, and the quality control machinery. Given the structural and functional similarities between yeast prions and proteins implicated in mammalian diseases, our studies here may provide additional framework to explore the pathways by which mutations lead to disease phenotypes.

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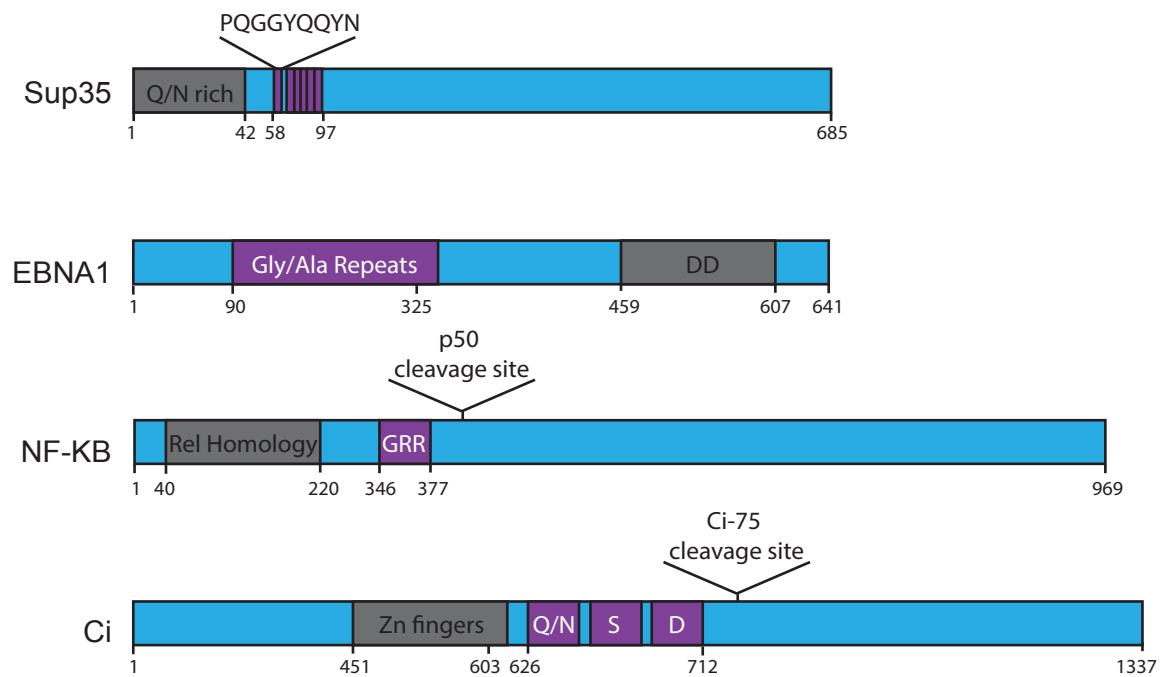


Figure1. Conserved domains of proteins with AAA+ ATPase processing defects. Regions of simple sequence are shown in purple, and tightly folded domains are shown in gray.

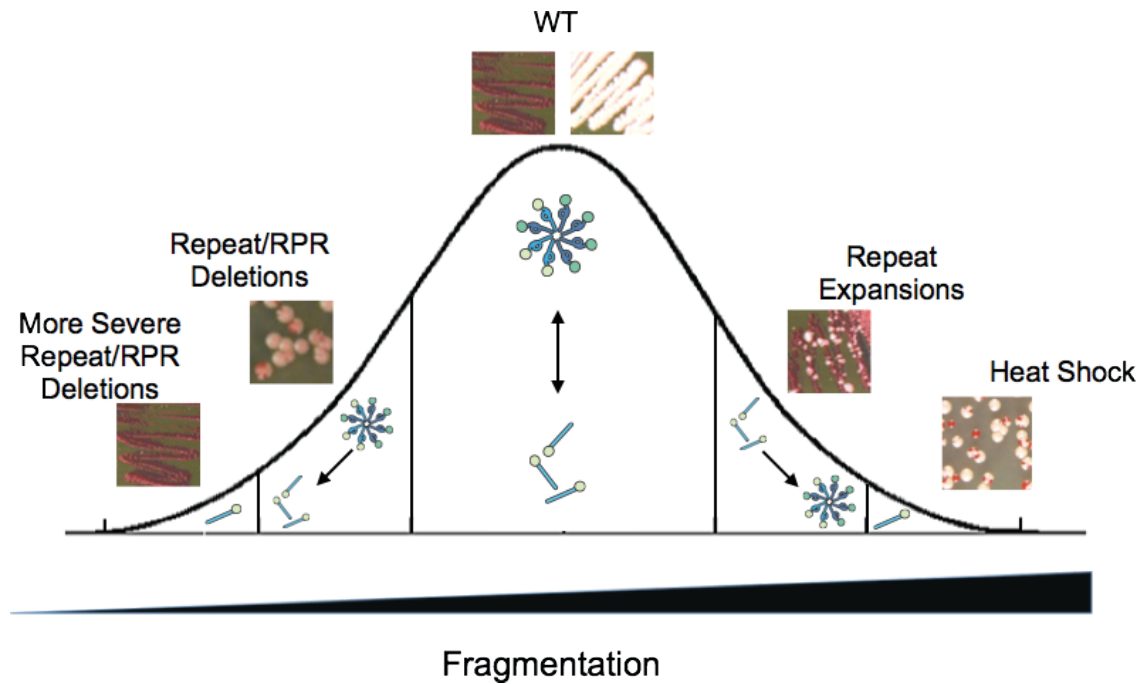


Figure 2. Precise levels of fragmentation are critically important for maintaining [PRION+] and [prion-] states. Increasing or decreasing fragmentation levels shifts the balance between prion assembly and dissolution, leading to prion loss.