

**The Mechanism of Dominant-Negative Prion Inhibition
Reveals the Crucial Interplay between Protein Misfolding and
its Cellular Environment**

By

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Chapter 1: Introduction

The Prion Phenomenon

The Prion Hypothesis

The prion hypothesis was originally formulated to explain the molecular basis of the scrapie agent, which causes fatal transmissible neurodegenerative disease in sheep and goats. This hypothesis posits that a unique group of proteins known as prions can act as transmissible epigenetic elements. These proteins self-replicate by adopting an alternative conformation which induces the conformational conversion of other molecules of the same protein into a like state (Griffith 1967; Prusiner 1982). This process requires that the prion agent can stably exist in at least two alternative conformations under physiological conditions: the self-replicating prion state and the normal cellular state. The term prion, for *proteinaceous infectious* particle was thus coined by Stanley Prusiner in 1982 to illustrate the protein-based composition of the scrapie agent (Prusiner 1982). In support of this proposal, the scrapie agent could be destroyed by protein-denaturing but not by nucleic acid-damaging conditions (Alper et al. 1966; Alper et al. 1967; Bellinger-Kawahara et al. 1987). Although the concept of a protein-only infectious agent was originally controversial, an abundance of evidence has accumulated in its support, including proof that recombinant prion protein converted to the self-replicating conformation *in vitro* can cause disease when introduced into mammals (Kim et al. 2010; Wang et al. 2010; Wang et al. 2011). Furthermore, self-replicating protein elements are now known to be responsible for a multitude of distinct biological phenomena, including several

transmissible diseases in mammals and heritable phenotypes in fungi (reviewed in: (Tuite and Serio 2010).

Remarkably, prions exhibit metastability, allowing for new traits to arise, endure, transmutate, or disappear within the lifetime of an individual. This metastability is governed by the relationship between the prion conformer with its in vivo propagation pathway, which encompasses multiple points to which slight modifications could dramatically alter the biological outcome. Studies on prion propagation in mammals and yeast have uncovered striking commonalities, demonstrating an ability to be similarly modulated by both cis and trans effectors. Understanding the mechanisms by which prion propagation is regulated should illuminate strategies for targeted intervention of prion diseases.

Mammalian Prion Diseases

Prion diseases naturally afflict several mammalian species including cow (Bovine Spongiform Encephalopathy - BSE), sheep (scrapie) and humans (Kuru, Creutzfeldt-Jakob disease, and others) (Table 1). Due to their infectious nature and their general sponge-like neuropathology, these diseases are collectively termed Transmissible Spongiform Encephalopathies (TSEs). Although there are unique symptoms and pathologies associated with each disease (see Prion Variants below), they are all progressively neurodegenerative, ultimately fatal, and associated with one conserved gene, PRNP, which encodes the prion protein (PrP) (Basler et al. 1986; Goldfarb et al. 1994; Prusiner 1994; Harrison et al. 2010).

While prion diseases may also arise in sporadic and familial forms, the major insight into the disease agent was gleaned from studies of prion infectivity. Prion infection may occur through many routes, including accidental or experimental inoculation with contaminated material or blood, or by ingestion of contaminated meat (Cuille and Chelle 1939; Will et al. 1996). While transmission of prion disease within the same species is most efficient, cross-species transmission is believed to occur naturally in rare circumstances and has been accomplished experimentally in laboratory mice and hamsters (Chandler 1961; Kimberlin et al. 1975; Kimberlin and Walker 1977; Will et al. 1996). A striking example of interspecies transmission is the appearance of BSE (mad cow disease), which is thought to have arisen by feeding scrapie contaminated offal to cattle (Wilesmith et al. 1988) and its subsequent transmission to humans, in the form of variant Creutzfeldt-Jakob Disease (vCJD), through the ingestion of BSE contaminated beef (Collinge et al. 1996; Bruce et al. 1997). The prion protein was identified as being the primary agent responsible for these diseases as it co-purified with infectivity (Bolton et al. 1982), and species-specific variations in PrP sequence are a primary component of the barrier to interspecies transmission (Prusiner et al. 1990). Direct proof of the protein-only mechanism of TSEs was recently provided from studies in mice, where recombinant PrP, existing in a self-replicating conformation, induced disease when inoculated into susceptible animals (Kim et al. 2010; Wang et al. 2010; Wang et al. 2011).

Fungal Prions

Along with causing a unique set of diseases in mammals, prions can serve as epigenetic determinants in fungi (Wickner 1994). Although fungal prions are not infectious per se, they are transmissible through cell division and mating. In contrast to mammals where the PrP protein is the only identified prion, there are several known transmissible prion proteins in fungi (Table 2). Indeed, the list of fungal prions continues to grow and includes proteins of various functions. Some of the best-studied examples are the *[URE3]*, *[PSI⁺]*, and *[PIN⁺]* prions of *Saccharomyces cerevisiae*, which are the prion forms of the Ure2, Sup35, and Rnq1 proteins, respectively (Cox 1965; Wickner 1994; Sondheimer and Lindquist 2000; Derkatch et al. 2001; Osherovich and Weissman 2001). Another well documented example is the *[Het-s]* prion of *Podospora anserina*, the prion form of the HET-s protein (Coustou et al. 1997).

While the molecular basis of how conformational conversion of PrP leads to prion disease is currently unknown (see Conformational Self-Replication below), conversion of fungal prion proteins to the self-replicating conformation results in the alteration of their normal cellular function and thereby new heritable phenotypes. For instance, Ure2 and Sup35, involved in nitrogen catabolism and translation termination respectively, display reduced activity when in their prion form, leading to the use of alternative nitrogen sources and stop codon readthrough respectively (Cox 1965; Lacroute 1971; Wickner 1994; Liebman and Derkatch 1999; Serio and Lindquist 1999; Wickner et al. 2001). Interestingly, Rnq1 has no known non-prion function, but when in the prion form, Rnq1 acquires a gain-of-function phenotype, which allows for the induction of other

yeast prions (Derkatch et al. 1997; Derkatch et al. 2000; Sondheimer and Lindquist 2000; Derkatch et al. 2001). Like Rnq1, the HET-s protein has no known non-prion function, but its prion form mediates heterokaryon incompatibility during cell fusion upon mating with a strain carrying the Het-S allele (Coustou et al. 1997). As is the case in mammals, transfer of self-replicating conformations of recombinant Ure2, Sup35, and Rnq1 protein into yeast leads to the appearance of $[URE3]$, $[PSI^+]$, and $[PIN^+]$, directly linking the heritable phenotypes to a protein-only mechanism (King and Diaz-Avalos 2004; Brachmann et al. 2005; Patel and Liebman 2007).

Conformational Self-Replication

While the transfer of a prion protein into a susceptible host clearly leads to a change in phenotype, the transmissibility of these phenotypes, whether through infectious or heritable routes, requires a mechanism for self-replication of that form within a living organism. Several methods of self-replication for a protein-only transmissible agent were suggested by Griffith in 1967. Of particular significance is a conformation-based model, in which an infectious protein binds to and stimulates the conformational conversion of another molecule of the same prion protein to the infectious state (Griffith 1967). A variety of *in vitro* and *in vivo* evidence now supports this prescient model, which is in line with our current understanding of prion-based self-replication. According to this model, prion replication begins with the initial misfolding of the normal prion protein, PrP^C (PrP-cellular), into the infectious state, PrP^{Sc} (PrP-scrapie). Misfolded PrP^{Sc} then associates with monomeric or soluble PrP^C and converts it to the misfolded PrP^{Sc}

form. Finally, the association of PrP^{Sc} molecules during the conversion process results in the formation and accumulation of higher ordered aggregates or complexes (Figure 1). Strikingly, studies both *in vivo* and *in vitro* have demonstrated that the underlying mechanism of conformational self-replication is similar for mammalian and yeast prion proteins.

Conformational Self-replication In Vitro

Self-replication of prion protein conformations can be reproduced *in vitro* using purified prion proteins. For instance semi-purified mammalian PrP^C converts to a protease-resistant form, believed to be related to PrP^{Sc}, *in vitro* upon the addition of PrP^{Sc}, which “seeds” the reaction (Kocisko et al. 1994). This *in vitro* process is typically very inefficient but can be drastically enhanced through a continuous cycle of sample incubation and sonication, a method termed protein misfolding cyclic amplification (PMCA) (Saborio et al. 2001), which generates additional templates for conversion by fragmenting larger aggregates into smaller complexes. Purified yeast prions can also misfold to form an aggregate *in vitro*, which occurs spontaneously after a lag phase or immediately upon the addition of preformed fibers or prion aggregate-containing lysates (Glover et al. 1997; Taylor et al. 1999; Serio et al. 2000). That this process represents the mechanism underlying prion replication *in vivo* and that prion fibers are responsible for prion phenotypes is supported by that fact that *in vitro* generated yeast prions induce the prion state when introduced into yeast cells (Maddelein et al. 2002; King and Diaz-Avalos 2004; Tanaka et al. 2004; Brachmann et al. 2005; Patel and Liebman 2007), and *in vitro* generated aggregates of the

mammalian prion protein create prion disease when inoculated into mice (Kim et al. 2010; Wang et al. 2010; Wang et al. 2011).

In Vivo Prion Biogenesis Cycle

While prion proteins clearly have the inherent capacity to adopt and self-replicate alternative conformations, emerging evidence suggests that the cellular environment plays an important role in modifying this process to create transmissible epigenetic elements *in vivo*.

Studies of the yeast prion Sup35 have been particularly instrumental in advancing our understanding of prion conformational self-replication within living cells. In addition to the conversion reaction that occurs *in vitro*, the self-replication of prion conformations *in vivo* involves a multi-step pathway of prion biogenesis that is mediated by cellular factors (Figure 2). The importance of these *in vivo* steps and the existence of interacting cellular factors on prion propagation most likely extends to all fungal prions and has been suggested to apply to the mammalian prion protein as well (Masel et al. 1999). Based on $[PSI^+]$ studies, four critical steps must occur to ensure the growth and transmission of prions within a population.

First, prion protein synthesis is clearly an important contributor to propagation of prion phenotypes. In mammals, PrP expression is required for disease (Bueler et al. 1993; Prusiner et al. 1993) the incubation period for clinical disease decreases upon increasing PrP expression (Prusiner, Scott et al. 1990; Tremblay, Meiner et al. 1998), and overexpressing PrP leads to a higher

frequency of prion disease (Scott et al. 1989; Sandberg et al. 2011). However, there is an imperfect correlation between PrP^{Sc} accumulation and clinical disease as mice with heterozygous disruptions of PrP show equal levels of PrP^{Sc} accumulation as their wild-type counterparts despite delayed clinical symptoms (Scott et al. 1989). In the yeast prion system, spontaneous prion induction is also stimulated by transient overexpression of yeast prion proteins (Chernoff et al. 1992; Chernoff et al. 1993; Derkatch et al. 1996), and overexpression of Sup35 is toxic to [PSI⁺] strains (Chernoff et al. 1992; Ter-Avanesyan et al. 1993; Derkatch et al. 1997), mirroring the enhanced toxicity (i.e. clinical disease) in mammals under the same conditions. Interestingly, overexpressing some yeast prions can also lead to prion loss (see below) (Derkatch et al. 1999; Allen et al. 2005).

Second, prion protein in the non-prion state must adopt the self-replicating conformation. As *in vitro*, this process occurs through a templating mechanism, in which existing aggregates of prion protein already in the form direct the remodeling the soluble protein coincident with its incorporation into those complexes. Both in mammals and in yeast, conversion occurs post-translationally. For example in mammals pulse labeled PrP is initially protease sensitive, but over a timescale of hours converts to a protease resistant state (Taraboulos et al. 1990; Caughey and Raymond 1991). While the [PSI⁺] prion in yeast has been shown to rapidly convert monomeric Sup35-GFP into the aggregated state when introduced into non-prion cells via mating (Satpute-Krishnan and Serio 2005).

Third, the aggregate template must be fragmented into smaller complexes to create additional surfaces for conversion. The crucial role of fragmentation in prion self-replication has previously been implicated *in vitro*, where sample agitation or sonication accelerates prion polymerization (DePace et al. 1998; Serio et al. 2000; Saborio et al. 2001; Gonzalez-Montalban et al. 2011), and several lines of evidence also demonstrate that fragmentation is critical *in vivo* for prion self-replication and transmission in both yeast and mammals (Masel and Jansen 2000). While the molecular mechanism by which prion aggregates are fragmented in mammals is currently unknown, in yeast, this role is filled by the molecular chaperone Hsp104 and its co-chaperones Hsp70 (Ssa1) and Hsp40 (Sis1) (Chernoff et al. 1995; Ness et al. 2002; Allen et al. 2005; Song et al. 2005; Satpute-Krishnan et al. 2007; Higurashi et al. 2008; Tipton et al. 2008). Hsp104 is essential for $[PSI^+]$ prion propagation as its deletion or inactivation results in prion loss (Chernoff et al. 1995; Eaglestone et al. 2000; Ferreira et al. 2001; Ness et al. 2002; Grimminger et al. 2004), and a direct role for Hsp104 in fragmentation is suggested by several studies. First, prion loss by Hsp104 inactivation causes propagons (heritable prion units or seeds) to decrease, while Hsp104 reactivation allows for propagon re-amplification (Bailleul et al. 1999; Eaglestone et al. 2000; Ness et al. 2002; Cox et al. 2003; Byrne et al. 2007). Furthermore, decreasing Hsp104 activity increases Sup35 aggregate size, while its reactivation rescues this increase in size (Wegrzyn et al. 2001; Kryndushkin et al. 2003). Additionally, experiments observing the fate of Sup35-GFP under different functional states of Hsp104 demonstrate that $[PSI^+]$ aggregates initially

continue to grow in the absence of Hsp104 activity but that soluble Sup35 eventually accumulates from the newly synthesized protein when templates become limiting (Bailleul et al. 1999; Satpute-Krishnan et al. 2007; Kawai-Noma et al. 2009).

Fourth, prion aggregates must be transmitted for progression of disease in mammals and for maintenance of the associated phenotype in a yeast population. In yeast, fragmentation of prion aggregates is also essential for efficient transmission. When Sup35 aggregates grow too large upon Hsp104 inactivation they become immobile and are preferentially retained in mother cells, leading to prion loss upon cell division (Bailleul et al. 1999; Eaglestone et al. 2000; Wegrzyn et al. 2001; Ness et al. 2002; Cox et al. 2003; Byrne et al. 2007; Satpute-Krishnan et al. 2007; Kawai-Noma et al. 2009). During the course of mammalian prion disease, PrP^{Sc} must spread to and throughout regions of the central nervous system to produce symptoms (Beekes et al. 1998). Although several mechanisms by which prions spread transcellularly have been suggested, it is possible that once prion aggregates are released to the extracellular space they can simply diffuse throughout neural tissue (reviewed in: (Beekes and McBride 2007; Aguzzi and Rajendran 2009). This could also explain the higher infectivity correlated with smaller prion aggregates, as they may have greater mobility (see: Modulating the Prion Cycle Affects Biological Outcomes) (Masel et al. 1999; Zampieri et al. 2009).

Together, these studies highlight each of the major steps in the conformational self-replication cycle *in vivo*. But how can we organize this information to explain

the biological manifestations of prion phenotypes? From one perspective, the conformational self-replication cycle can be viewed as a pathway of prion aggregate biogenesis, with aggregates playing a central role in the establishment of prion-associated phenotypes. Consistent with this idea, the aggregation, and thereby depletion of active protein from the soluble pool, correlates with the loss-of-function phenotypes of some yeast prions, including Sup35 and Ure2. Moreover, mammalian prion diseases correlate with the presence of the misfolded form of PrP and the deposition of PrP aggregates in neural tissue (Prusiner 1998). In this case, however, it is not known whether PrP aggregates themselves (Silveira et al. 2005), an oligomeric intermediate, or some lethal byproduct of their formation, is the cause of neurotoxicity (reviewed in: (Caughey and Lansbury 2003; Collinge and Clarke 2007). Regardless of the crucial form, the efficiency of the conformational self-replication cycle will dictate the steady-state accumulation of all possible species of the prion protein and thereby the associated phenotype. Given the inextricable links to prion aggregates, understanding how the physical properties of these complexes affects the efficiency of their self-replication *in vivo* is key to understanding prion biology.

Prion Amyloid Structure

Misfolding into the prion form is most often associated with an increased β -pleated sheet content and increased resistance to proteolytic degradation, either from an originally alpha helical-rich protein (for the mammalian PrP) or an unstructured prion domain (fungal prions) (Prusiner 1982; Caughey et al. 1991; Pan et al. 1993; Taylor et al. 1999; Balbirnie et al. 2001). To date, all known prion

proteins propagate in an amyloid-like formation, self-assembling into amyloid-like fibrils *in vitro* (Glover et al. 1997; Taylor et al. 1999; Legname et al. 2004; Wickner et al. 2008) and forming amyloid-like deposits or aggregates *in vivo* (Paushkin et al. 1996; Dos Reis et al. 2002). Amyloid fibrils display enhanced protease resistance, bind dyes such as Congo red, and produce birefringence under polarized light (Glenner 1980; Glenner 1980; Sipe and Cohen 2000). Through EM, fibers appear unbranched, of varying length, and relatively straight, while X-ray fiber diffraction typically reveals a predominantly cross- β structure, with β -sheets perpendicular to the fiber axis and hydrogen bonding between strands parallel to the fiber axis (Sunde and Blake 1997; Serpell et al. 2000; Makin and Serpell 2005).

Several proteins are capable of forming amyloid-like fibers, although prions are the only ones classically considered infectious. Non-infectious amyloid formation is also associated with disease (e.g. amyloid deposits of the β -amyloid protein (A- β) in Alzheimer's) or with a useful biological function (e.g. amyloids of the curli protein of *E. coli*,) (Chapman et al. 2002), (reviewed in: (Vetrivel and Thinakaran 2006; Baxa 2008; Maury 2009). It may be that differential cellular mechanisms or structural elements separate infectious from non-infectious amyloid forming proteins, however the precise delineation between these classes is not clearly understood (reviewed in: (Sigurdsson et al. 2002; Baxa 2008). Moreover, this distinction may not be absolute, as at least A- β amyloid can induce cerebral amyloidosis via peripheral inoculation in mice (Eisele et al. 2010), and many neurodegenerative diseases associated with protein misfolding are thought to

spread throughout the brain through cell-to-cell infection, leading to their designation as prionoids (reviewed in: (Brundin et al. 2010). Therefore, studying prions may provide insight not only into the structure but also into the replication and transmission of other amyloid forming proteins.

The structure of prion fibrils may additionally shed light on their mechanism of conformational self-replication. Indeed, the fibrillar nature of prion complexes suggests a templating process whereby normally folded forms of the protein first bind to fiber ends, convert into the fiber-compatible form, and become stably incorporated, giving each fiber only two growth-competent surfaces. Furthermore, the structure of the fiber might suggest the degree of unfolding the native protein must first undergo to assemble into the prion state or elucidate how changes in the primary sequence of the prion protein might affect the fibers properties. However, due to the difficulty of studying amyloid-like aggregates through conventional methods the precise arrangements of amino acids within prion fibers remain debatable. β -sheets could be arranged in several different orientations: parallel in-register, parallel out of register, antiparallel, or β -helix (Perutz et al. 2002). For instance, X-ray diffraction and NMR analysis of short peptides have shown β -strands bonding together in a complimentary steric zipper, with opposing side chains interdigitating to exclude water and stabilized through van-der-waals attractions (Nelson et al. 2005; Sawaya et al. 2007; van der Wel et al. 2007). However, a parallel in-register structure with identical residues from aligning monomers stacking upon one another has been proposed by solid-state NMR studies with larger prion fragments (Diaz-Avalos et al. 2005)

and by the demonstration that prion formation, while influenced by overall amino acid content, is somewhat independent of the linear sequence of amino acids (Ross et al. 2004; Ross et al. 2005; Ross et al. 2005). Additionally, a β -helix, with β -sheets forming rings around a water filled central pore, has been suggested through solvent accessibility experiments (Krishnan and Lindquist 2005) and X-ray diffraction (Kishimoto et al. 2004). Furthermore, although an amyloid (i.e. cross- β) conformation has been indicated by several studies of prion fibers, this feature may not be universal. For instance, Ure2 fibrils have shown several properties which are inconsistent with a cross- β conformation and suggest instead that these fibers are mostly alpha-helical, with monomers within the fiber retaining much of their native and soluble characteristics (Bousset et al. 2002; Bousset et al. 2003; Bai et al. 2004).

Interestingly, the atomic structures from fibers composed of short Sup35-derived peptides revealed several different classes of steric zippers, demonstrating the ability of these peptides to stack in different orientations (Sawaya et al. 2007). Thus, the interactions between monomers within a prion fiber may not necessarily produce a single stable structure. Indeed, conformational diversity is suggested by prion phenotypic variability and evidenced by differential biochemical properties displayed by prion aggregates composed of identical protein (see Prion Variants).

Prion Variants

Rather than causing a distinct syndrome, the misfolding of PrP has been linked to a family of related disorders, with unique phenotypes, in mammals. Although this variability was initially difficult to reconcile with a protein-based infectious element, as genetic mutations seemed a more plausible explanation, several studies on mammalian and yeast prions explain phenotypic variability through conformational flexibility of the prion protein. This flexibility allows prions to adopt a spectrum of self-replicating conformations (prion variants), which assemble into aggregates with discrete core amyloid structural properties. As might be predicted by the pathway of prion propagation, the properties of these prion complexes impact their efficiency of self-replication and thereby their associated phenotypes *Mammalian Prion Variants*

Variability of prion disease phenotypes was first recognized experimentally when scrapie from sheep was transmitted and serially passaged in goats, giving rise to two discrete lines that consistently produced stereotypical clinical symptoms, “drowsy” or “scratching”, due to the characteristic behavior of the infected animals (Pattison and Millson 1961). Prion variants have now been observed in a wide range of natural and experimental settings, but the most extensive information on mammalian prion variants has come from work on inbred mouse lines. Through these, and other mammalian prion studies, it is apparent that prion variants are distinguishable by a variety of distinct and repeatable disease characteristics. The most obvious clinical difference among variants is the incubation time between prion inoculation and illness onset, which is strikingly consistent when the same variant is inoculated into inbred mice (Pattison and

Millson 1961; Dickinson and Fraser 1969; Dickinson and Meikle 1969; Outram 1976; Bruce et al. 1991; Bessen and Marsh 1992). Other distinguishing characteristics include the type, distribution, and severity of pathological changes in the brain, location of amyloid or protease-resistant PrP deposits, ease of interspecies transmission, and resistance to heat inactivation (Fraser and Dickinson 1973; Fraser 1976; Dickinson and Taylor 1978; Kimberlin and Walker 1978; Carp et al. 1984; Bruce et al. 1989; Bruce et al. 1991).

In addition to disease variations in laboratory animals, naturally occurring human prion infections, such as kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), and variant Creutzfeldt-Jakob disease (vCJD), also differ in their clinical characteristics. For instance, CJD presents with memory loss and confusion that progresses to severe dementia, while FFI presents as intractable insomnia that may persist for several months until other symptoms occur, generally sparing cognitive function until late in the time course of disease (Brown and Mastrianni 2010). As would be expected for distinct behavioral abnormalities, the neuropathology also differs in stereotypical ways, similar to those mentioned for mouse prion variants (Parchi et al. 1996; McLean et al. 1998; McLean 2008). Furthermore differences between PrP^{Sc} proteolytic sensitivity correlates with different syndromes (Parchi et al. 1996). Overall, these diseases may represent unique prion variants, in addition to, or dictated by, changes in PrP sequence (see: Prion Polymorphisms Modulate Phenotype).

As predicted by the prion hypothesis, phenotypic differences between prion variants are specified by physical differences in the prion protein. For example, prion variants can be biochemically characterized by their distinct sensitivity to proteases and protein denaturants (i.e. conformational thermodynamic stability) (Bessen and Marsh 1992; Bessen and Marsh 1994; Bessen et al. 1995; Safar et al. 1998; Legname et al. 2006; Ayers et al. 2011). Interestingly, for mouse prion variants, amyloid thermodynamic stability is inversely related to disease severity, with less thermodynamically stable variants displaying shorter incubation times (Safar et al. 1998; Legname et al. 2006). This intriguing link between aggregate thermodynamic stability and prion-associated phenotype was initially interpreted as a change in the rate of conformational self-replication, primarily mediated through differences in aggregate frangibility. However, Transmissible mink encephalopathy variants display the reverse trend in the hamster model system (Bessen and Marsh 1992; Bessen and Marsh 1992; Bessen and Marsh 1994; Ayers et al. 2011), perhaps suggesting a more complicated interplay between aggregate stability and prion-associated phenotype within a cellular context (see Modulating the Prion Cycle Alters Biological Outcomes, below).

Yeast Prion Variants

Fungal prion variants have also been classified and extensively studied for $[PSI^+]$, $[URE3]$, and $[PIN^+]$ (Derkatch et al. 1996; Schlumpberger et al. 2001; Bradley et al. 2002; Brachmann et al. 2005; Edskes et al. 2009). Interestingly, $[Het-s]$ variants have never been observed, and it could be possible that HET-s

can only exist in one stable prion conformation. The $[PSI^+]$ prion system in particular has been especially useful for the study of prion variants.

As mentioned earlier, the yeast prion Sup35 functions as an essential release factor that aids in translation termination (Stansfield et al. 1995; Zhouravleva et al. 1995). In the prion form ($[PSI^+]$), Sup35 is aggregated, and its function is compromised. In the non-prion form ($[psi^-]$), Sup35 is soluble and efficiently terminates translation (Cox 1965; Patino et al. 1996; Paushkin et al. 1996). Phenotypically, $[PSI^+]$ and $[psi^-]$ cells can be distinguished by the suppression of certain nonsense alleles such as *ade1-14* (Cox 1965). The premature stop codon of *ade1-14* is read through in $[PSI^+]$ cells, producing full length Ade1 protein and white colonies on rich media. In contrast, $[psi^-]$ *ade1-14* cells produce a truncated Ade1 protein, resulting in red colonies on rich media due to the accumulation of a metabolic intermediate in the adenine biosynthetic pathway (Figure 3) (Fisher 1969). The state of Sup35 can be directly visualized *in vivo* using a Sup35-GFP fusion, or examined using biochemical techniques that separate soluble and aggregated Sup35 (Patino et al. 1996; Paushkin et al. 1996). $[PSI^+]$ is metastable, with a low frequency of both mitotic loss and spontaneous induction (Cox et al. 1980; Lund and Cox 1981). Consistent with its self-replicating nature and cytoplasmic localization, $[PSI^+]$ is dominant upon mating and segregates in a non-Mendelian fashion, producing four $[PSI^+]$ meiotic progeny (Cox 1965).

In addition to the non-prion and prion states, $[PSI^+]$ variants are also readily detectable based on their phenotype. $[PSI^+]$ can be induced in yeast cells, dependent on the presence of another yeast prion $[PIN^+]$, by the overexpression

of Sup35 (Chernoff et al. 1992; Derkatch et al. 1997). Upon induction of $[PSI^+]$, individual prion inductants vary in their stop codon readthrough efficiency and mitotic stability, suggesting phenotypic variability analogous to mammalian prion variants (Derkatch et al. 1996). $[PSI^+]$ variants are often referred to as “strong” or “weak” for their respective degrees of phenotypic severity, i.e. stop codon readthrough and epigenetic stability. The phenotypic severity of $[PSI^+]$ is a product of the level of functional (soluble) Sup35 available for readthrough (Zhou et al. 1999; Tanaka et al. 2006) (Figure 3b). Therefore, weaker variants of $[PSI^+]$ appear pink on rich media, do not grow as efficiently as strong variants on media lacking adenine, and have higher frequencies of mitotic prion loss.

Discrete $[PSI^+]$ phenotypes can also be produced by introducing *in vitro* generated Sup35 fibers of distinct conformations into yeast cells (King and Diaz-Avalos 2004; Tanaka et al. 2004; Toyama et al. 2007). Furthermore, placing physical constraints on the Sup35 protein, such as crosslinking different regions, directs the assembly of specific fibers *in vitro*, which when introduced into yeast, give rise to specific $[PSI^+]$ phenotypes (Krishnan and Lindquist 2005). Interestingly, fibers produced *in vitro* can also be influenced to adopt different conformations depending on the temperature at which they were assembled. Prion fibers assembled at 4°C (Sc4) contain a shorter region protected from hydrogen-deuterium exchange and are smaller than those assembled at 37°C (Sc37), suggesting a less extensive amyloid core and thermodynamic stability for the former (Tanaka et al. 2006; Toyama et al. 2007). Despite being less thermodynamically stable, Sc4 fibers introduced into yeast give rise to a strong

prion phenotype, while Sc37 fibers induce a weak phenotype (Tanaka et al. 2006). Consistent with these *in vitro* analyses, Sup35 aggregates from cell lysates of weak and strong prion variants also differ in their thermodynamic stabilities, displaying differential resistance to heat denaturation, with strong aggregates again being more susceptible to denaturation and thus less thermodynamically stable (Pezza et al. 2009). These studies collectively demonstrate that the physical conformation of the prion within the aggregate is predictably interpreted *in vivo* to generate a distinct biological outcome.

Modulating the Prion Cycle Alters Biological Outcomes

Given the physical differences among prion variants, it is straightforward to predict that aggregates composed of these variants direct distinct biological outcomes by modulating various steps within the self-replication cycle *in vivo*. But, predicting the precise ways in which these alterations will manifest as new phenotypes is particularly complicated given the interdependency of each step in the pathway.

For example, physical changes in prion aggregates can lead to changes in the rate of self-replication and therefore the abundance of these complexes *in vivo* (Tanaka et al. 2006). According to this “abundance-based” model, the equilibrium between soluble and aggregated protein determines the cellular phenotype by dictating the concentration of the species that confers the prion-associated phenotype (i.e. soluble Sup35 in the case of $[PSI^+]$). Consistent with this model, prion fibers that fragment more easily *in vitro* (i.e. are less

thermodynamically stable) give rise to a strong $[PSI^+]$ state by directing the rapid incorporation of soluble Sup35 *in vivo*, a process that is limited by the availability of aggregate templates (Ness et al. 2002; Tanaka et al. 2006; Satpute-Krishnan et al. 2007). Furthermore, prion variant-specific phenotypes (i.e. different soluble/aggregated Sup35 ratios) can be mathematically modeled by adjusting the kinetics of different *in vivo* steps (Sup35 synthesis, conversion, fragmentation) (Tanaka et al. 2006).

A similar mathematic approach has been used to explain mammalian prion variants, interpreting the *in vitro* measurement of fiber stability as a difference in fiber amplification *in vivo* (Zampieri et al. 2009). Although this model includes several parameters, fibril breakage is again found to be of paramount importance. As previously mentioned, mouse prion variants display an inverse correlation between thermodynamic stability and disease severity (i.e. incubation period length) (Legname et al. 2006). Thus, more frangible fibers should enhance prion self-replication, explaining the shorter incubation periods.

The abundance-based model, though informative, has been found to be incomplete through another computational approach modeling the Sup35/ $[PSI^+]$ system and experimental testing of the predictions generated (Derdowski et al. 2010). Although the abundance-based model could account for differential readthrough phenotypes of $[PSI^+]$ variants, it could not reproduce other defining characteristics such as prion loss frequency (Derkatch et al. 1996; Byrne et al. 2009), differential aggregate size distributions (Kryndushkin et al. 2003; Bagriantsev et al. 2006), and the observation that prion loss occurs more

frequently upon Sup35 overexpression (Derkatch et al. 1999; Allen et al. 2005). In contrast, the abundance-based model predicted completely stable prion states because aggregates were always abundant enough to direct inheritance of the associated phenotype. To capture all known experimental aspects of prion variants, an additional parameter, placing a size-based threshold on prion aggregate transmission, was required. Interestingly, prion variant characteristics were faithfully reproduced when the transmissible size limit fell within the range that delineates weak from strong $[PSI^+]$ aggregates biochemically (Kryndushkin et al. 2003).

Several inferences could be made by this model and experimentally tested. First, propagons could be defined as representing only the subset of aggregates below the transmissible size limit, with variants differing in the amount of propagons available for transmission due to their distinct range of aggregate sizes. This differential accumulation of propagons in weak and strong variants would therefore explain their respective frequencies of spontaneous prion loss. Secondly, experimentally shifting the aggregate size range should result in a corresponding shift in spontaneous prion loss. Finally, heterogeneity should arise within a population due to the differential accumulation and transmission of aggregates, with mother cells retaining larger and more aggregates than daughters. These predictions were each validated experimentally utilizing a variety of methods. For instance, Sup35-GFP aggregate transmission to daughter cells during budding was shown to be less efficient for weak variants utilizing a fluorescence loss in photobleaching (FLIP) assay; increasing prion

aggregate size by Sup35 overexpression increased prion mitotic instability, and finally readthrough efficiency, propagon numbers, and aggregate sizes stereotypically differed in mother and daughter cells demonstrating population heterogeneity (Derdowski et al. 2010).

Can information provided from the size-based model of the $[PSI^+]$ yeast prion system apply to mammalian prion disease, despite the obvious differences between a dividing yeast culture and a post-mitotic neuronal population? As previously mentioned, the thermodynamic stability (fragmentation efficiency) of PrP aggregates influences its converting activity and infectivity (Legname et al. 2005; Legname et al. 2006; Zampieri et al. 2009). Enhanced fragmentation would not only result in increased aggregate number but also create smaller aggregates which have been correlated with higher converting activity and infectivity in the mammalian prion systems (Silveira et al. 2005; Calvez et al. 2009; Tixador et al. 2010). However, it is difficult to disentangle the mechanistic basis of the enhanced infectivity of small aggregates as this could simply be the result of enhanced frangibility (and thus higher proliferation rates). However, it has been suggested that the increased infectivity of smaller aggregates could be due to their ability to diffuse more easily throughout neural tissue (Masel et al. 1999; Zampieri et al. 2009). Consistent with this idea, more thermodynamically stable, and presumably larger, aggregates are associated with localized pathology while less thermodynamically stable aggregates are associated with more widespread pathology (Legname et al. 2005).

As is the case for yeast prions, mammalian prion biology is also altered in

different ways by changes in prion expression. As mentioned earlier, the incubation time for clinical disease, but not for the generation of prion infectivity, is highly dependent on PrP expression level (Scott et al. 1989; Sandberg et al. 2011). One way to explain this observation is that infectivity increases with aggregate abundance until reaching a plateau, with the plateau representing a limitation on the system such as fragmentation activity. From this point, aggregates will increase in size but not number, with growth proceeding more rapidly at higher PrP expression. Prion toxicity could be the result of larger and more toxic aggregates, or from the titration of other cellular factors through their association with aggregates, a mechanism that has been proven for the toxicity of overexpressed Sup35 in $[PSI^+]$ yeast cells (Vishveshwara et al. 2009). Thus, it is possible that a size-based mechanism of infectivity is an underappreciated factor in determining the phenotypic outcomes of prion variants in mammals.

It is important, however, to remember that the link between prion variant thermodynamic stability and prion severity is not always congruent. As previously mentioned, in the hamster model system TME variants display a reverse trend; variants with the least stability actually have the longest incubation periods (Bessen and Marsh 1992; Bessen and Marsh 1992; Bessen and Marsh 1994; Ayers et al. 2011). This suggests that although fragmentation is clearly necessary for prion self-replication and transmission there may also be an upper boundary on this process. The long incubation of these less stable variants appears to be a product of inefficient prion conversion and higher rates of prion clearance *in vivo* (Shikiya et al. 2010; Ayers et al. 2011). Indeed, prion clearance

has been demonstrated in mice (Safar et al. 2005), and established prion infections can be reversed through inhibition of new PrP expression (Mallucci et al. 2003; Mallucci et al. 2007; White et al. 2008) presumably through clearance of prion amyloids, highlighting the potential for competition between prion replication and prion clearance.

Prion-associated phenotypes thus arise through a dynamic pathway of prion biogenesis, and alterations of the steps within this pathway can characteristically manifest as distinct biological outcomes. As prion variants can simultaneously modulate multiple steps of the *in vivo* prion propagation cycle their global behavior must be understood in order to accurately explain their effects on cellular physiology. The information gleaned from these studies has led to a more holistic understanding of prion biology and provided explanations for several previously anomalous observations. Can this framework be extended to explain other complex aspects of prion biology? In order to address this question we can examine other conditions known to modify prion phenotypes. By determining the mechanisms whereby other conditions alter prion biology we can thus continue to advance our understanding of *in vivo* prion biogenesis.

Prion Polymorphisms Modulate Phenotype

A range of prion sequence polymorphisms have been identified that impact the biological outcome of prion infections and the susceptibility to prion disease. Several PrP polymorphisms or gene mutations are correlated with enhanced vulnerability to, or conversely protection from, prion disease, and fungal prion

mutations or insertions have also been shown to modulate the prion-associated phenotype. These prion protein sequence variations may be exerting differential effects on the *in vivo* prion propagation pathway, making them an avenue through which this pathway may be further explored.

PrP Genotype and Prion Disease

A genetic component of mammalian prion diseases has been implicated in many situations. The human prion diseases CJD, GSS, and FFI often occur as familial diseases, consistent with an autosomal dominant pattern of inheritance (Collinge and Palmer 1994). The heritable component of these diseases suggested that there was a genetic predisposition, and CJD, GSS, and FFI cases have since been linked to the PRNP locus (Hsiao et al. 1989; Owen et al. 1989). Several general phenotype-genotype correlations can be made for the major subtypes of these diseases. GSS was first associated with a proline to leucine substitution at position 102, but there are a number of point (including premature stop-codons) and insertional mutations now reported in affected individuals for each disease (reviewed in: (Brown and Mastrianni 2010).

The common Val/Met polymorphism at coding position 129 also plays an important role in prion susceptibility and phenotypic outcome (Owen et al. 1990; Palmer et al. 1991). The genotype at this position affects the clinical symptoms of prion diseases, the pattern of protease-resistant PrP deposits in the brain, and the biochemical properties of PrP^{Sc} aggregates (proteolytic processing) (Kitamoto et al. 1992; de Silva et al. 1994; MacDonald et al. 1996; Parchi et al. 1997; Hauw

et al. 2000). Homozygosity at codon 129 appears to be a risk factor for prion disease, accounting for more rapid disease progression and the majority of kuru and CJD cases, with Met 129 homozygotes showing increased susceptibility (Collinge et al. 1991; Palmer et al. 1991; Collinge et al. 1996; Will et al. 1998; Will et al. 2000; Lee et al. 2001; Mead et al. 2003; Jeong et al. 2005). Interestingly this Val/Met polymorphism also determines the disease outcome when in combination with another genetic mutation D178N. The D178N mutation is correlated with CJD when there is a Met at 129, while it is associated with FFI when coupled with 129V (Goldfarb et al. 1992).

The presence of mutations that correlate with prion diseases suggests that differences in prion sequence may kinetically shift the prion protein towards a misfolded or amyloid state. Furthermore, the fact that different mutations and polymorphic combinations underlie distinct diseases, suggest that certain sequences might favor distinct prion variant conformations, with those variants giving rise to unique pathology through modulation of the *in vivo* prion propagation pathway.

Sequence/Variant Interactions mammalian PrP

Although it was shown that scrapie could be infectious via artificial introduction in the 1930's, a genetic aspect still seemed to play a role in susceptibility to the disease in sheep and goats (Parry 1962; Gordon 1966; Dickinson 1976; Hunter et al. 1997). For instance, some sheep never showed symptoms of disease within several years of being challenged by a frequently used scrapie variant,

indicating a genetic component to disease protection (Dickinson et al. 1968). A similar genetic element underlying resistance to prions was observed in the mouse scrapie model system and displayed differential relationships with scrapie variants. These studies began when it was discovered that one partially inbred mouse line displayed a significantly longer incubation time to the onset of encephalopathy than another partially inbred line after challenged with the mouse adapted scrapie variant ME7 (Dickinson and Mackay 1964). The gene responsible for this difference was designated sinc (scrapie *incubation*) or Prn-i (*Prion incubation*) depending on the group, with the two genotypes designated as sinc^{s7}/Prn-p^a or sinc^{p7}/Prn-p^b to denote short or *prolonged* incubation of ME7 (Dickinson and Meikle 1969; Kingsbury et al. 1983). Similar genotype/variant interactions were displayed for several other mouse adapted scrapie, CJD, and BSE variants (Kingsbury et al. 1983; Carlson et al. 1986; Bruce et al. 1991; Bruce et al. 1994). Interestingly, some prion variants displayed the reverse trend, having shorter incubation periods in sinc^{p7} than sinc^{s7}. However, after the initial inoculation with a long incubation genotype/variant combination, subsequent passages to the same genotype resulted in a consistent and shorter incubation period, demonstrating adaptation of the variant to the host genotype (Carlson et al. 1989). Furthermore, heterozygotes of the two lines displayed intermediate or longer incubation periods than either of the two homozygous lines (Dickinson and Mackay 1964; Dickinson et al. 1968). Since these initial observations, genetic analysis has revealed that the sinc/Prn-i gene is analogous to the prion protein gene, PRNP (Carlson et al. 1986; Race et al. 1990; Hunter et

al. 1992; Moore et al. 1998). Targeted gene alterations of PRNP demonstrate that L108/T189 (sinc^{s7}/Prn-p^a) F108/V189 (sinc^{p7}/Prn-p^b) dimorphisms underlie this unique genotype/variant interaction, controlling incubation time and modulating prion disease features (Moore et al. 1998).

Sequence/Variant Interactions [PSI⁺]

Sup35 is composed of three domains: a C-terminal domain encoding the essential GTPase that functions in translation termination, a highly charged middle domain of unknown function, and the N-terminal prion-forming domain (Ter-Avanesyan et al. 1993; Tuite and Cox 2003). The prion-forming domain is crucial for converting Sup35 into the prion state *in vivo* and for the formation of amyloid fibers *in vitro* (Paushkin et al. 1996; Glover et al. 1997; King et al. 1997). The extreme N-terminus, amino acids 1-39, is composed of an unusually high (>50%) density of glutamine and asparagine residues (glutamine asparagine rich, or QNR, region), while residues 40-112 contain an oligopeptide-repeat (OPR) region composed of five and a half degenerate repeats (Figure 4) (Kikuchi et al. 1988; Kushnirov et al. 1988; Wilson and Culbertson 1988; Ter-Avanesyan et al. 1993; Liu and Lindquist 1999).

Single amino acid and deletion mutations in the prion-forming domain of Sup35 influence the ability of the protein to adopt self-replicating conformations and provide insight into the function of subdomains within this region. Both the QNR and OPR regions are necessary for [PSI⁺] aggregation and maintenance, with studies suggesting that the QNR tract predominantly directs homotypic

interactions to induce aggregation and that the OPR region influences the inheritance of these aggregates through an unknown mechanism (DePace et al. 1998; Liu and Lindquist 1999; Santoso et al. 2000; Borchsenius et al. 2001; Parham et al. 2001; Osherovich et al. 2004). Intriguingly, these mutations reveal variant-specific interactions with $[PSI^+]$, as is the case for host-pathogen interactions in mammals. For instance, the expression of some point mutants of Sup35 display altered degrees of phenotypic severity in different variants (i.e. readthrough efficiency), reflecting the efficiency of their incorporation into existing prion aggregates (King 2001; Chang et al. 2008). Therefore, utilizing a library of Sup35 point mutants can actually distinguish subtle conformational differences among phenotypically similar variants by reporting on their unique patterns of interaction (King 2001; Chang et al. 2008).

Sequence as a species barrier

The existence of a species barrier to prion disease has long been noted, as prion infections rarely spread from one species to another. However, as strikingly demonstrated by the recent bovine spongiform epidemic and its subsequent transmission to humans (vCJD) (Collinge et al. 1996; Bruce et al. 1997), this barrier is not absolute. Indeed, these diseases have sparked renewed interest in understanding the mechanisms which restrict or permit interspecies prion transmission. The interspecies barrier phenomenon appears to be predominantly a product of the PrP primary sequences of host and donor protein and the conformation of the donor PrP^{Sc} amyloid.

The importance of compatibility among PrP sequences is demonstrated by loss of the normal resistance to hamster or mink prions displayed by mice (Marsh et al. 1969; Kimberlin and Walker 1978; Taylor et al. 1986) upon transgenic expression of hamster or mink PrP respectively (Scott et al. 1989; Windl et al. 2005). The role of the prion conformation in establishing transmission barriers is suggested by seed/monomer identity dependence on heterologous *in vitro* prion conversions. For instance, preformed Syrian hamster PrP fibers nucleate the conformational conversion of mouse PrP, but mouse PrP fibers cannot nucleate Syrian hamster amyloid formation (Vanik et al. 2004). However, when mouse PrP was first seeded by Syrian hamster fibers, they could then be used to nucleate the Syrian hamster PrP, suggesting that they adopted a conformation with which the Syrian hamster PrP sequence was now compatible (Vanik et al. 2004).

Although prion interspecies barriers can manifest as a seemingly complete block of prion transmission, they can also display adaptability. For instance, first passages of semi-incompatible prions to new species can result in a lengthened incubation period while on subsequent passages to the same species the incubation period is characteristically shortened (as is described for mammalian genotype/variant interactions above) (Carlson et al. 1986). The passaging of prions through different host species appears to create or select for new prion variants, as this process has been shown to give rise to final prion isolates with characteristics that differ from the original inoculate (Pattison and Jones 1968; Dickinson 1976; Kimberlin et al. 1989; Peretz et al. 2002). Interestingly, passaging a prion through a new species can produce a variant with reduced

thermodynamic stability and incubation period, further demonstrating the link between these properties as previously discussed (Legname et al. 2006). Prion variants can also be passaged between different species *in vitro* using amyloid seed and monomeric PrP of different species. *In vitro* passaging of amyloid fibers with monomeric PrP of a new species creates amyloids that have increased infectivity to the new species *in vivo* (Castilla et al. 2008; Green et al. 2008).

Prion species barriers are also well documented for the yeast [*PSI*⁺] system and mimic many of the observations made in the mammalian system. For instance, [*PSI*⁺] species barriers demonstrate asymmetry (Chen et al. 2010) and variant dependency (Tanaka et al. 2005; Chen et al. 2007; Vishveshwara and Liebman 2009; Afanasieva et al. 2011), while species specificity is influenced by the sequence used for initial seeding (Chien and Weissman 2001). Furthermore, expression of cross-species sequences can generate novel prion variants (Chien and Weissman 2001; Chien et al. 2003; Tanaka et al. 2005) as well as cause prion loss (Chen et al. 2007; Afanasieva et al. 2011).

For [*PSI*⁺], highly divergent Sup35 sequences display a strong species barrier which appears to restrict heterologous Sup35 sequences from co-aggregating (Kushnirov et al. 2000; Santoso et al. 2000). In contrast, more closely related Sup35 sequences are often capable of co-aggregation, however a barrier to efficient prion propagation still occurs among some sequence/variant combinations (Chen et al. 2007; Chen et al. 2010). The QNR tract and OPR region have been implicated in mediating the barrier between divergent and more closely related species, respectively (Santoso et al. 2000; Chien and Weissman

2001; Chen et al. 2010), suggesting that the QNR region might restrict the initial co-aggregation between species (Santoso et al. 2000; Chien and Weissman 2001), while the oligopeptide-repeat region may mediate the conversion of aggregates into heritable species (Chen et al. 2010). Indeed, there is evidence that although cross-species protein can bind to heterologous aggregates they are inefficiently converted into the polymer (Chen et al. 2010). Interestingly, prion loss induced by co-expression of incompatible sequences could be explained by aggregate growth impairment due to monomer binding accompanied by inefficient conversion, although alteration of other aggregate properties may also explain loss.

Together this information suggests that the sequence of the prion protein restricts the spectrum of self-replicating conformations accessible to the protein. Barriers to cross-species prion transmission would effectively be caused by the inability of the host prion to be converted to, or maintained by, the conformation of the introduced amyloid, with certain incompatible interactions inducing prion loss through an unknown mechanism(s). In contrast, cross-species barriers would be overcome if the host PrP was capable of adopting (or adapting) the conformation of the introduced prion variant.

Prion Inhibition and Resistance

The sequence of a prion protein appears not only to restrict its range of adoptable conformations, and thereby its ability to replicate certain variants, but also to dominantly inhibit prion self-replication by wild-type protein *in vivo*.

Dominant-negative inhibition of wild-type prion self-replication via expression of heterologous prion proteins is not only an extreme example of prion phenotypic switching but is also surprising given that prion propagation is normally quite efficient. As such, dominant-negative conditions may inform our understanding of how modulation of the prion propagation pathway can lead to drastic phenotypic alterations, as well as suggest therapeutic interventions for prion diseases.

The Met/Val polymorphism of the human prion sequence plays a role in prion protection, the mechanism of which has been suggested by *in vitro* studies, where mixtures of partially unfolded Met129 and Val129 PrP assemble into a non-amyloid off-pathway intermediate, which slows progression to the amyloid state (Baskakov et al. 2002; Tahiri-Alaoui et al. 2004; Tahiri-Alaoui et al. 2006). Two other common polymorphisms have been identified that confer some prion disease protection: Glu/Lys 219 in humans (Gln/Lys 218 mouse) and Gln/Arg 171 in sheep (Gln/Arg 167 mouse), with basic residues associated with sCJD and scrapie resistance respectively (Goldmann et al. 1994; Westaway et al. 1994; Belt et al. 1995; Clouscard et al. 1995; Ikeda et al. 1995; Bossers et al. 1996; van Keulen et al. 1996; Hunter et al. 1997; O'Rourke et al. 1997; Shibuya et al. 1998). Transgenic mouse, tissue culture, and *in vitro* studies of these protective alleles demonstrate a dominant-negative effect on prion formation of the heterologous protein (Kaneko et al. 1997; Perrier et al. 2002; Crozet et al. 2004; Furuya et al. 2006; Lee et al. 2007; Hizume et al. 2009). Furthermore, although endogenous PrP and extra-species or chimeric PrP protein in transgenic mice are prion competent alone, they can inhibit prion disease when

co-expressed, thereby also exerting dominant-negative inhibition *in vivo* (Telling et al. 1994; Priola and Chesebro 1995; Telling et al. 1995). Given their dominant effects, it seems likely that these sequence variants perturb some aspect(s) of the prion propagation pathway outlined above; thereby serving as a system to further probe prion self-replication *in vivo*.

Several studies using dominant-negative mutants have provided us with some clues as to their potential mechanism(s) of inhibition. Initially, incompatible PrP proteins were proposed to compete for an unidentified co-factor, essential for prion conversion (protein x) (Telling et al. 1995; Kaneko et al. 1997; Zulianello et al. 2000). However, the necessity of the co-factor in mediating the dominant-negative effect of these alleles was later ruled out as inhibition of PrP^{Sc} formation could be accomplished by these mutants in cell-free systems (Lee et al. 2007; Geoghegan et al. 2009). Thus, these dominant-negative proteins appeared to be acting on the prion fiber itself.

An alternative to the protein x model, proposed through mathematical modeling, suggested that wild-type and heterologous PrP could incorporate into the same fiber and alter its properties (Masel and Jansen 2000). For example, they could act as “end-blocking drugs”; precluding the additional binding or conversion of heterologous protein onto the fiber and thereby being effective at lower ratios, or, they could affect other aspects of prion self-replication such as fragmentation, requiring incorporation throughout the complex and thereby higher effective concentrations (Masel and Jansen 2000). Consistent with this suggestion, dominant-negative prion mutants differ in their effective inhibitory ratios relative to

the wild-type or heterologous protein (Priola et al. 1994; Kaneko et al. 1997; Priola 1999; Horiuchi et al. 2000; Lee et al. 2007; Geoghegan et al. 2009). For instance, the dominant negative Q218K protein inhibits the wild-type prion only at high ratios (Lee et al. 2007; Geoghegan et al. 2009), while Q171R acts at sub-stoichiometric ratios (Geoghegan et al. 2009).

[PSI⁺]-No-More in yeast

Deletions and single amino acid changes in both the QNR tract and the OPR region of Sup35 have been found to alter the behavior of the [PSI⁺] prion (Doel et al. 1994; DePace et al. 1998; Kochneva-Pervukhova et al. 1998; Derkatch et al. 1999). Like the dominant-negative inhibition observed for mammalian prion polymorphic alleles, some point mutants of Sup35 mutants dominantly inhibit the propagation of wild-type [PSI⁺] when constitutively or transiently co-expressed. Interestingly, like the Q219K and Q171R polymorphisms of PrP, virtually all mutants associated with [PSI⁺] inhibition resulted in a change from an uncharged to a charged residue at key positions (DePace et al. 1998). Because of their ability to permanently revert the [PSI⁺] state these mutants are typically termed PNM, for [PSI⁺]-No-More (Doel et al. 1994). Given their dominant effects and the tractability of the yeast prion model system, PNM mutants are an invaluable resource with which to refine our understanding of how modulation of the prion propagation pathway can drastically affect the biological outcome of prion alternative conformations.

Although PNM mutants have been characterized by other labs, their behavior was sometimes puzzling. For instance, a well-studied PNM mutant, Gly58Asp (G58D or PNM2), supports $[PSI^+]$ when expressed alone but displays variant-specific interactions with $[PSI^+]$, eliminating strong $[PSI^+]$ but enhancing the severity and stability weak $[PSI^+]$ (Kochneva-Pervukhova et al. 1998; Derkatch et al. 1999) Furthermore, *in vitro* studies of the yeast $[PSI^+]$ dominant-negative mutant G58D were confounding in that this mutant was able to convert normally in some circumstances but not in others (Kochneva-Pervukhova et al. 1998; Osherovich et al. 2004). The available data were therefore incapable of explaining the molecular mechanism of $[PSI^+]$ curing by PNM proteins.

Given our evolving insight into the mechanisms underpinning *in vivo* prion propagation, it is plausible that dominant-negative $[PSI^+]$ mutants are exerting their effects by altering distinct events in the prion propagation pathway, as predicted through mathematical modeling of prion propagation in mammals (Masel and Jansen). The experimental tractability of the yeast system would allow a direct test of this concept for the first time; therefore, I took a holistic *in vivo* approach to elucidate the mechanisms of inhibition exerted by two dominant-negative Sup35 mutants, Q24R and G58D. Both mutants result in a change to a charged residue in the QNR tract or OPR two of Sup35 respectively. These mutants were chosen for further study based on their history (G58D) and strength (preliminary results with Q24R) and their potential to help define the function of the QNR tract and the OPR region. In order to mechanistically explain their inhibitory effects *in vivo*, I examined their effects on the steps of the

prion biogenesis cycle and defined the conditions whereby these mutants became effective inhibitors. This also involved illuminating their relationship with molecular chaperones, specifically Hsp104. This work illustrates that the cellular environment mediates the establishment and propagation of prion-associated phenotypes and provides mechanistic insight into prion sequence/variant interactions. Furthermore, it not only reasserts the importance of the multifaceted *in-vivo* prion propagation pathway, but enhances it.

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Table 1: Mammalian Prion Diseases

Disease	Host	Cause
familial Creutzfeldt-Jakob disease (fCJD)	Human	Genetic: D178N-129V, V181I, T183A, T188K, T188R-129V, E196K, e200K, V203I, R208H, V210I, E211Q, M232R, octarepeat insertions
sporadic Creutzfeldt-Jakob disease (sCJD)	Human	unknown
variant Creutzfeldt-Jakob disease (vCJD)	Human	Infection
Gerstmann-Straussler-Sheinker disease (GSS)	Human	Genetic: P102L-129M, P105-129M, A117V-129V, G131V-129M, Y145*-129M, H197R-129V, F198S-129V, D202N-129V, Q212P, Q217R-129M, M232, octarepeat insertions
Fatal Familial Insomnia (FFI)	Human	Genetic: D178-129M
Kuru	Human	Infection
Scrapie	Sheep and goats	Infection
Bovine spongiform encephalopathy (BSE)	Cattle	Infection
Transmissible mink encephalopathy (TME)	Mink	Infection
Chronic wasting disease (CWD)	Deer, elk, and moose	Infection
Feline spongiform encephalopathy (FSE)	Cats	Infection

Adapted from Benetti and Legname, 2009

Table 2: Fungal Prions

Prion State	Protein	Function	Phenotype
[<i>PSI</i> ⁺]	Sup35	Translation termination	Nonsense suppression
[<i>URE3</i>]	Ure2	Transcriptional co-repressor	Faulty nitrogen catabolite repression
[<i>PIN</i> ⁺]	Rnq1	Unknown	Allows <i>De novo</i> prion induction
[Het-s]	HET-s	Unknown	Heterokaryon incompatibility
[<i>OCT</i> ⁺]	Cyc8	Transcriptional co-repressor	Derepression of transcription
[<i>MOT3</i> ⁺]	Mot3	Transcriptional co-repressor	Derepression of transcription
[<i>SWI</i> ⁺]	Swi1	Subunit of SWI-SNF chromatin remodeling complex	Repression of transcription
[<i>MCA</i> ⁺]	Mca1	Metacaspase, regulates apoptosis	None observed
[<i>ISP</i> ⁺]	Sfp1	Transcriptional activator	Translational accuracy

Adapted from Tuite and Serio, 2010

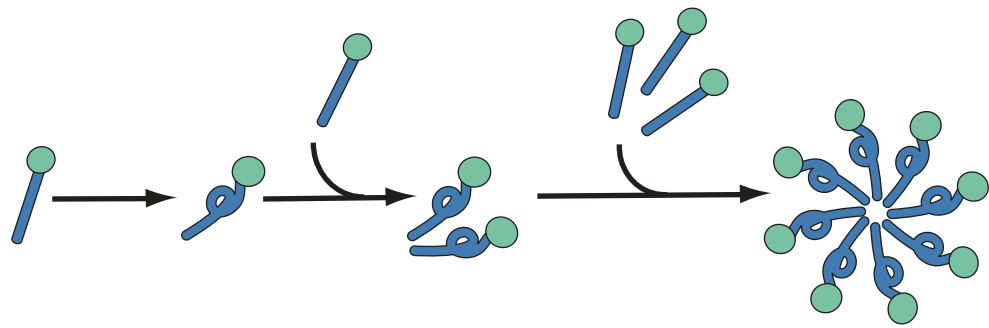


Figure 1. Protein conformational replication. Prions can exist in two stable conformations, the non-prion form (straight blue line) or the misfolded prion form (curly blue line). Once a protein misfolds into a prion form, it templates the conversion of the normally folded protein into the prion form through physical association, leading to the formation of ordered prion complexes/aggregates.

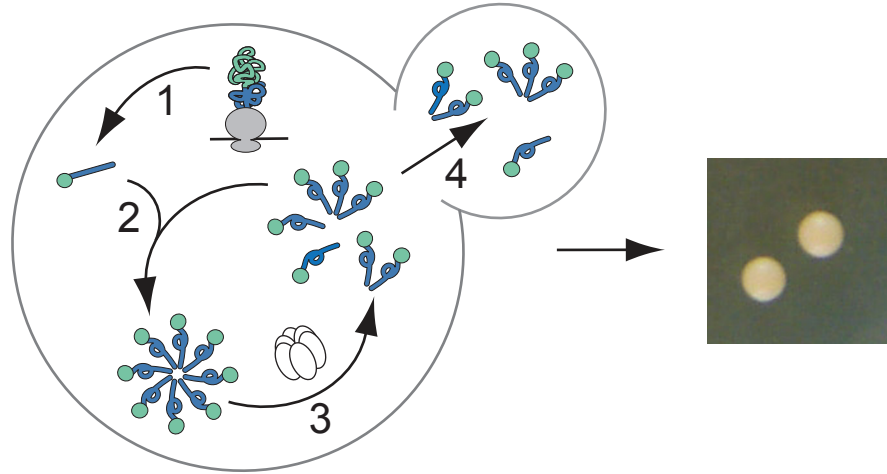


Figure 2. Yeast prion *in vivo* biogenesis cycle. Self-replicating protein conformations create stable phenotypes (white colonies) *in vivo* when the processes of synthesis (1), conversion (2), fragmentation (3) by Hsp104 (hexamer) and transmission (4) are balanced to allow aggregates of prion proteins to persist in the cellular population.

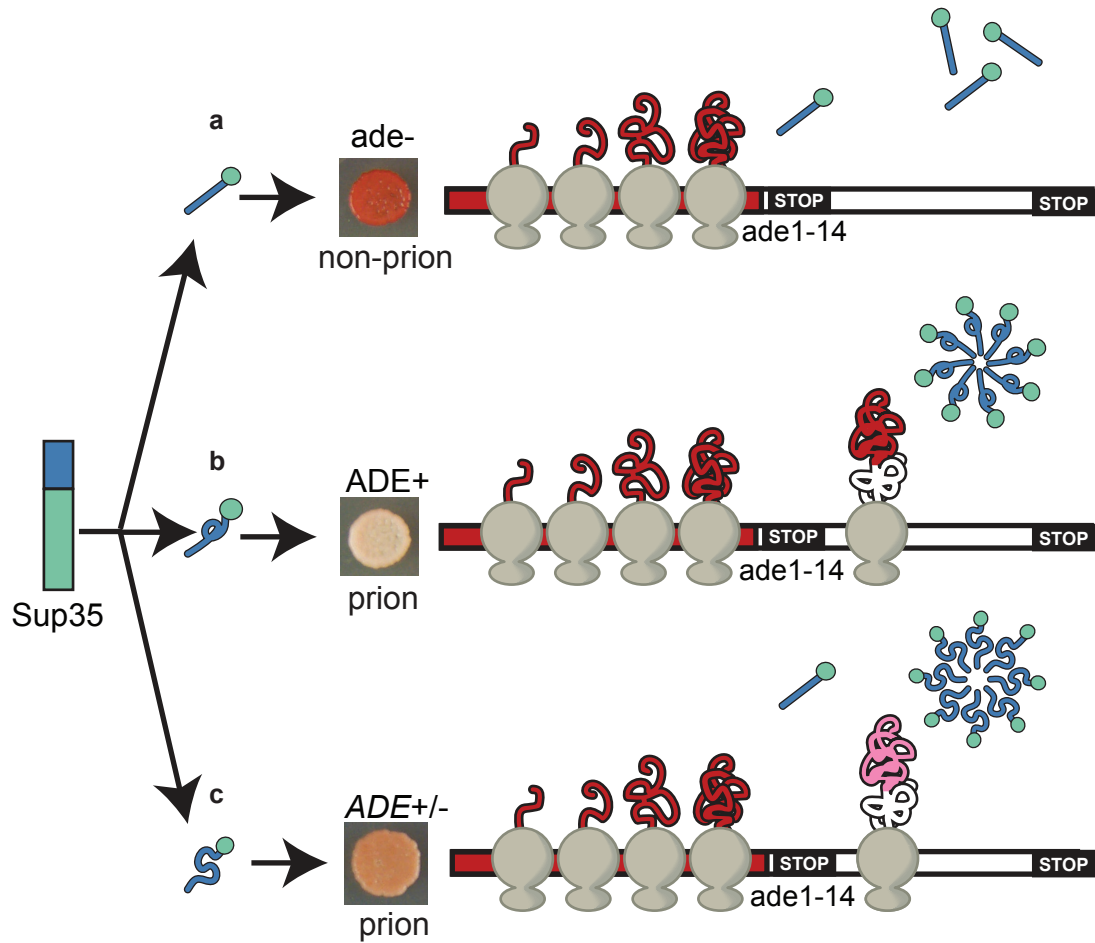


Figure 3. Sup35/[PSI⁺] Prion. Sup35 can exist in a non-prion [*psi*⁻] (a) and prion [PSI⁺] form (b and c), which can be distinguished phenotypically by the suppression of certain non-sense alleles, based on the extent to which Sup35 function is sequestered in aggregates dictated by the underlying prion variant. The premature stop codon of *ade1-14* is read through in [PSI⁺] cells, producing full length Ade1 protein and white (b) or pink (c) colonies on rich media for strong or weak prion variants, respectively. In contrast, [*psi*⁻] *ade1-14* cells (a) produce a truncated Ade1 protein, resulting in red colonies on rich media.

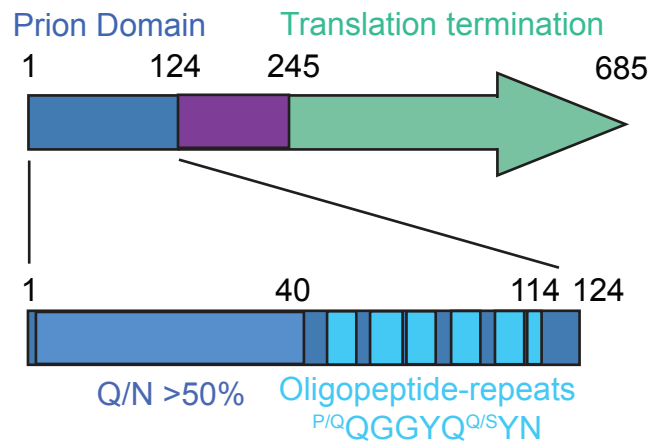


Figure 4. Sup35 is composed of three domains: a C-terminal domain encoding the essential GTPase that functions in translation termination, a highly charged middle region of unknown function, and the N-terminal prion-forming domain (Ter-Avanesyan, Kushnirov et al. 1993; Tuite and Cox 2003). The prion domain, amino acids 1-39, is composed of an unusually high (>50%) density of glutamine and asparagine residues, while residues 40-112 contain an oligopeptide repeat region composed of five and a half degenerate repeats.

Chapter 2: Dominant Prion Mutants Induce Curing Through Pathways That Promote Chaperone-Mediated Disaggregation

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I performed all the experiments except the FLIP (Fig5c, Fig6) and the relative fluorescence intensity of DsRed zygotes (Fig3c.).

Abstract

Protein misfolding underlies many neurodegenerative diseases, including the Transmissible Spongiform Encephalopathies (prion diseases). While cells typically recognize and process misfolded proteins, prion proteins evade protective measures by forming stable, self-replicating aggregates. However, co-expression of dominant-negative prion mutants can overcome aggregate accumulation and disease progression through currently unknown pathways. Here, we determine the mechanisms by which two mutants of the *Saccharomyces cerevisiae* Sup35 protein cure the $[PSI^+]$ prion. We show that both mutants incorporate into wildtype aggregates and alter their physical properties in different ways, diminishing either their assembly rate or their thermodynamic stability. While wildtype aggregates are recalcitrant to cellular intervention, mixed aggregates are disassembled by the molecular chaperone Hsp104. Thus, rather than simply blocking misfolding, dominant-negative prion mutants target multiple events in aggregate biogenesis to enhance their susceptibility to endogenous quality control pathways.

Introduction

Cellular phenotypes are established, in large part, by the collective activities of their unique proteomes, and alterations to the physical state of individual proteins, such as changes in conformation, have the capacity to induce dramatic phenotypic switches. The prototypical conformation-based phenotypes are those associated with prion proteins, which have been implicated in a number of distinct biological phenomena including transmissible neurodegenerative diseases in mammals, heritable traits in fungi, and synaptic efficiency in an invertebrate (Tuite and Serio 2010). These changes in cellular function, however, can only emerge if the misfolded prion proteins persist and are amplified to levels that perturb normal cellular homeostasis. Accumulation of misfolded prion proteins is facilitated by their assembly into ordered aggregates, which not only protect these aberrant species from refolding or degradation but also template the conversion of other forms of the protein to a like state (Tuite and Serio 2010). This conformational self-replication, along with the continuous fragmentation of aggregates into smaller complexes, mediates an exponential increase in misfolded prion protein over the course of disease (Masel et al. 1999).

The efficient and autocatalytic pathway of prion protein misfolding provides a molecular explanation for the progressive nature of prion diseases, but it also complicates their effective treatment, which would require the complete resolution of existing aggregates. This challenge, however, may not be insurmountable. Naturally occurring polymorphisms in the mammalian prion protein PrP alter the pathological and clinical characteristics of prion diseases

(Tuite and Serio 2010). In most of these cases, heterozygosity at the PrP locus mitigates the severity of disease (Baker et al. 1991; Gambetti et al. 1995; Cervenakova et al. 1998; Deslys et al. 1998; Huillard d'Aignaux et al. 1999; Collinge et al. 2006; Webb et al. 2008). In addition, differences in prion protein sequence confer strain-dependent resistance to infection and to the transmission of disease between species,(Dickinson et al. 1968; Carlson et al. 1988; Goldmann et al. 1994; Westaway et al. 1994; Belt et al. 1995; Clouscard et al. 1995; Ikeda et al. 1995; Bossers et al. 1996; Hunter et al. 1997; O'Rourke et al. 1997; Shibuya et al. 1998; Baylis et al. 2002) and co-expression of some allelic combinations reduces or eliminates the accumulation of misfolded PrP *in vivo* (Kaneko et al. 1997; Perrier et al. 2002; Crozet et al. 2004; Furuya et al. 2006; Toupet et al. 2008). Intriguingly, the severity of these dominant-negative effects is dependent upon the relative stoichiometry of mutant and wildtype protein, and different mutants can be distinguished by the ratios at which they become effective inhibitors (Perrier et al. 2002; Atarashi et al. 2006; Geoghegan et al. 2009).

Given the central role of misfolded prion protein in the appearance, maintenance and spread of prion-associated phenotypes, dominant-negative prion mutants are thought to inhibit the self-replicated misfolding of wildtype prion protein. In this scenario, the observed ratio effects can be explained as differences in affinity, with the mutants inhibiting the conversion of wildtype prion protein to the misfolded form through direct binding (Kaneko et al. 1997; Lee et al. 2007; Geoghegan et al. 2009). However, distinct effective inhibitory ratios may also

indicate that individual mutants interfere with prion propagation through unique pathways. For example, mutants that target the conversion step are predicted to act efficiently at substoichiometric levels given the limited number of templating surfaces on an aggregate, while mutants that alter fragmentation or transmission of aggregates may act only at higher concentrations where they incorporate into these complexes at levels sufficient to alter their properties (Masel and Jansen 2000).

Here, we use the Sup35/[PSI⁺] system of *Saccharomyces cerevisiae* to uncover the mechanisms by which dominant-negative prion mutants inhibit prion propagation *in vivo*. [PSI⁺] is a self-replicating misfolded (prion) form of the translation release factor Sup35 (Tuite and Serio 2010). Under normal conditions, Sup35 is synthesized and rapidly converted to the prion form in a [PSI⁺] strain by interaction with and incorporation into existing aggregates (Satpute-Krishnan and Serio 2005). The molecular chaperones Hsp104, Hsp70 (Ssa1) and Hsp40 (Sis1) must then fragment these aggregates, by extracting and unfolding constituent Sup35 monomers, to generate additional templates for conversion and to ensure their efficient transfer to daughter cells (Chernoff et al. 1995; Ness et al. 2002; Satpute-Krishnan et al. 2007; Higurashi et al. 2008; Tessarz et al. 2008; Tipton et al. 2008; Kawai-Noma et al. 2009). This misfolding partially compromises Sup35 function, leading to a defect in translation termination that can be easily scored in yeast strains encoding genes with nonsense mutations. For example, [PSI⁺] strains carrying the *ade1-14* allele form white colonies on rich and adenine-deficient media due to readthrough of a

premature stop codon in the *ADE1* open reading frame, but strains with defective prion propagation, or those that have lost the prion state ($[psi^-]$), form red colonies on rich medium and fail to grow in the absence of adenine due to the accumulation of active, non-prion state Sup35 (Chernoff et al. 1995).

For these studies, we focused on two dominant-negative prion mutants, the $[PSI^+]$ -no-more (PNM) variants, Q24R and G58D (also known as PNM2) (Young and Cox 1971; Doel et al. 1994; DePace et al. 1998). These substitutions map to two different elements in the bipartite Sup35 prion-determining domain, the glutamine and asparagine-rich region (QNR, amino acids 1-40) and the oligopeptide-repeat region (OPR, amino acids 41-97), respectively, which are both necessary for the stable inheritance of the $[PSI^+]$ prion (Tuite and Serio 2010). Although both mutants dominantly induce prion curing, we show that they do so by targeting distinct events in the Sup35 misfolding pathway. In both cases, these changes impact aggregate dynamics *in vivo*, ultimately promoting chaperone-mediated disassembly of existing aggregates.

Results

Effective inhibitory ratios distinguish PNM mutants

The dominant-negative effect of G58D on $[PSI^+]$ propagation has been reported in some yeast strains but not others (Doel et al. 1994; Derkatch et al. 1999). These differences may reflect distinct interactions of the mutant with each genetic background, but the previous studies also differed in the expression level of the mutant relative to wildtype Sup35. To explore the possibility that PNM

mutants inhibit prion propagation in a dose-dependent manner, we generated diploid $[PSI^+]$ yeast strains expressing wildtype Sup35 at different ratios relative to the PNM mutants G58D and Q24R (2:1, 1:1, 1:2; see Fig. 1). Expression of G58D at any ratio in a $[PSI^+]$ strain eliminated growth on adenine-deficient medium, indicating reversal of the prion phenotype (Fig. 2a). By colony color, the severity of this effect increased with G58D dosage, with a wildtype-to-mutant ratio of 1:2 displaying a colony phenotype that was indistinguishable from the $[psi^-]$ state (Fig. 2a). The dominant-negative effect of Q24R expression on the $[PSI^+]$ colony phenotype also increased with its dosage, but the prion colony color phenotype was suppressed even at the substoichiometric ratio (2:1 wildtype-to-mutant; Fig. 2a).

To determine if reversal of the $[PSI^+]$ phenotype upon PNM expression reflected prion loss (curing), we analyzed $[PSI^+]$ inheritance in the wildtype meiotic progeny of these strains. For G58D, $[PSI^+]$ inheritance was largely stable at the substoichiometric (0% curing) and equal ratios (3% curing), where the colony color phenotype was only mildly reversed (Fig. 2a, b). However, $[PSI^+]$ did not appear in wildtype meiotic progeny when G58D was expressed in excess of wildtype Sup35 in the parental diploid (1:2 wildtype-to-mutant; 100% curing; Fig. 2b), consistent with the complete reversal of the prion phenotype (Fig. 2a). For Q24R, which had a more severe effect on the prion phenotype (Fig. 2a), 30% of wildtype meiotic progeny lost $[PSI^+]$ even at the substoichiometric ratio (2:1 wildtype-to-mutant), and this instability increased with its dosage (Fig. 2b). Thus, $[PSI^+]$ curing in diploids expressing PNM mutants parallels the severity of the

phenotypic reversal in these strains, and PNM mutants, like their mammalian counterparts, can be distinguished by their effective inhibitory ratios.

PNM mutants target multiple events in prion propagation

The distinct effective inhibitory ratios for G58D and Q24R may reflect differences in affinity for a single event in the Sup35 misfolding pathway or, alternatively, interference with different steps in this process. To distinguish between these possibilities, we first determined whether G58D or Q24R expression altered conversion of Sup35 to the prion form by analyzing their ability to incorporate into wildtype aggregates *in vivo*. In haploid strains, wildtype and PNM Sup35 did not interact in non-prion [*psi*⁻] cells, as expected, but both the G58D and Q24R proteins were specifically co-immunoprecipitated with HA-tagged wildtype Sup35 in [*PSI*⁺] cells (Fig. 3a). Previous studies *in vivo* have demonstrated G58D aggregation in [*PSI*⁺] strains (Derkatch et al. 1999), but studies *in vitro* disagree on whether this variant can efficiently convert to the prion form (Kochneva-Pervukhova et al. 1998; Osherovich et al. 2004). To assess the efficiency of G58D and Q24R conversion to the prion form *in vivo*, we monitored the appearance of stop codon readthrough in zygotes formed by mating a [*psi*⁻] strain expressing a fluorescent reporter of translation termination efficiency (GST-UGA-DsRed) to a [*PSI*⁺] strain. Production of fluorescent protein reflects the quantitative and rapid incorporation of soluble Sup35 into aggregates and the concomitant reduction in its activity (Satpute-Krishnan and Serio 2005). In a cross between G58D [*psi*⁻] and wildtype [*PSI*⁺] strains, the frequency of fluorescent zygotes was similar to a wildtype cross (~80%, Fig. 3b); however, the

intensity of this fluorescence was ~20% lower in the mutant cross (Fig. 3c), consistent with a previous study suggesting a mild conversion defect for G58D (Kochneva-Pervukhova et al. 1998). In contrast, we observed fluorescence in only ~40% of zygotes resulting from a cross between Q24R [*psi*⁻] and wildtype [*PSI*⁺] strains, indicating a strong conversion defect for Q24R *in vivo* (Fig. 3b).

Next, we determined whether incorporation of PMN Sup35s into wildtype aggregates altered the efficiency with which they are fragmented *in vivo*. Because this process is altered by the thermodynamic stability of aggregates (Tanaka et al. 2006), we assessed the sensitivity of these complexes, isolated from haploid strains expressing a 1:1 wildtype-to-mutant ratio (see Fig. 4a, b), to disruption with 2% (w/v) SDS at different temperatures (Satpute-Krishnan and Serio 2005). Expression of G58D did not alter the fraction of total soluble Sup35 recovered at 50°C, where aggregates remain intact (Fig. 3d) (Satpute-Krishnan and Serio 2005). However at both 60°C and 70°C, the amount of total soluble Sup35 increased by a factor of ~2.5 in comparison with a wildtype strain (Fig. 3d), indicating destabilization of the aggregates by G58D incorporation. For a strain expressing Q24R, a similar increase in total soluble Sup35 was detected at all temperatures in comparison with a wildtype strain (Fig. 3d), suggesting that Q24R expression did not alter the thermodynamic stability of aggregates but rather led to the accumulation of soluble Sup35 *in vivo*. Thus, the different effective inhibitory ratios of the PNM mutants correspond to alterations of distinct events in the Sup35 misfolding pathway *in vivo*, with G58D primarily altering the fragmentation step and Q24R affecting the conversion step.

PNM mutants alter propagon accumulation but not transmission

Modulation of the rates of conversion and fragmentation correlate with differences in the size, number, and transmissibility of aggregates *in vivo* and thereby the stability of prion-associated phenotypes (Derdowski et al. 2010). To determine the mechanism(s) by which PNM mutants dominantly induce $[PSI^+]$ loss, we assessed the effects of their expression on each of these parameters.

To determine the aggregate size distribution, we analyzed lysates of haploid strains expressing both wildtype and G58D or Q24R Sup35s by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) (Kryndushkin et al. 2003). For strains expressing either mutant, the size distribution of SDS-resistant aggregates was shifted to smaller complexes relative to wildtype strains (Fig. 5a). This effect was more severe for Q24R, consistent with the enhanced frequency of prion loss (Fig. 2b) and increased accumulation of soluble Sup35 in strains expressing this mutant in comparison with G58D (Fig. 3d). Similar effects were also observed in strains expressing a Q24K but not a Q24N or a Q24A allele (Fig. 4b-d), suggesting that positive charge at this position mediated the dominant inhibition.

To determine if these changes in aggregate size correlated with the number of heritable prion units (propagons), we genetically determined these values for haploid strains expressing an extra copy of wildtype, Q24R or G58D *SUP35* (Fig. 4a, b) using an *in vivo* dilution assay (Cox et al. 2003). For the wildtype strain, the median number of propagons was ~100 (Fig. 5b), and expression of Q24R

and G58D had opposing effects on this baseline. For strains expressing Q24R, the median number of propagons decreased by a factor of ~ 7 (Fig. 5b), consistent with both the decreased proportion of aggregated Sup35 (Fig. 3d) and the increased frequency of $[PSI^+]$ loss (Fig. 2b). In contrast, the median number of propagons increased by a factor of ~ 2 in the G58D strain relative to the wildtype strain (Fig. 5b). This observation is consistent with the idea that G58D incorporation increases the efficiency of aggregate fragmentation, but it also indicates that the increased frequency of $[PSI^+]$ loss in this strain (Fig. 2b) cannot be explained by a failure to generate sufficient propagons.

To determine if G58D induces prion curing by altering the efficiency of aggregate transmission, we used a fluorescence loss in photobleaching (FLIP) assay in strains expressing Sup35-GFP-labeled aggregates (Derdowski et al. 2010). For this analysis, we continually bleach either mother or daughter cells prior to cytokinesis, and monitor loss of fluorescence in the other cell as a proxy for protein transmission (Fig. 6). Using this assay, aggregates containing wildtype Sup35-GFP are transmitted with similar efficiencies in either direction in the presence or absence of G58D (Fig. 5c), indicating that the dominant-negative effect of this mutant also does not result from a defect in aggregate transmission.

PNM mutants promote Hsp104-mediated disassembly of aggregates

The increase in propagons and the efficient transfer of aggregates to daughter cells are difficult to reconcile with an increased frequency of $[PSI^+]$ loss for strains expressing G58D. However, we considered the possibility that the changes in

aggregate accumulation and stability induced by PNM expression (Fig. 3d) altered their interactions with other cellular components. We uncovered the molecular defects in prion propagation in haploid strains expressing both wildtype and PNM Sup35s (Figs. 3, 5), a mild over-expression of the prion protein relative to other cellular components. To determine if this imbalance impacted PNM effects, we assayed the number of propagons in diploid strains expressing either two copies of wildtype *SUP35* or one copy each of wildtype and PNM *SUP35* (Fig. 7a) to maintain proper expression ratios. Rather than inducing an increase in propagons as in haploids (Fig. 5b), G58D expression in diploids resulted in a reduction in propagons by a factor of ~7.5 (Fig. 8a). This enhanced inhibition of prion propagation in diploids was also apparent in a strain expressing Q24R, where the number of propagons decreased more dramatically in the diploid than in the haploid strain relative to wildtype (decrease by a factor of ~30 vs. ~7; Fig. 5b, 8a). Together, the strong effects of dosage on propagon accumulation suggested that other cellular factors could be mediating the biological outcomes of PNM mutant expression.

The best-characterized *trans*-regulator of $[PSI^+]$ propagation is the molecular chaperone Hsp104. To determine if imbalance between Sup35 and Hsp104 could explain the dosage effects on PNM efficacy, we constructed heterozygous disruptions of *HSP104* in diploid strains expressing two copies of wildtype *SUP35* or one copy each of wildtype and PNM *SUP35* (Fig. 7a, b) to approximate our experiments in haploids. Disruption of a single copy of *HSP104* decreased the number of propagons in a wildtype strain by factor of ~1.5, consistent with its

catalytic role in fragmentation (Fig. 8a) (Ness et al. 2002; Satpute-Krishnan et al. 2007). In contrast, heterozygous disruption of *HSP104* in strains expressing either G58D or Q24R increased the number of propagons by factor of ~3-4 (Fig. 8a) and partially reversed the phenotypic effects of PNM expression (Fig. 2a), suggesting that an excess of Hsp104 activity mediated PNM inhibition of prion propagation *in vivo*. Because PNM expression did not alter Hsp104 protein levels (Fig. 7b,c), we hypothesized that this increase in apparent Hsp104 activity arose because it was simply functioning as a better enzyme. Specifically, the Q24R conversion defect limits the production of aggregates (Fig. 3d) and thereby skews the substrate-to-enzyme ratio, while the G58D-mediated decrease in aggregate stability (Fig. 3d) enhances fragmentation.

To experimentally determine the relationship between Hsp104 activity and PNM dominant inhibition, we compared aggregate size, as a proxy for Hsp104-mediated fragmentation efficiency, (Kryndushkin et al. 2003; Tanaka et al. 2006) with suppression of the $[PSI^+]$ phenotype under various conditions. In a strain expressing a 2:1 wildtype-to-G58D ratio, heterozygous disruption of *HSP104* (Fig. 7c) decreased the mobility of aggregates by SDD-AGE (Fig. 8b and Fig. 9a), suggesting that Hsp104 activity was limiting, and this limitation suppressed the reversal of the $[PSI^+]$ phenotype (Fig. 2a). In contrast, heterozygous disruption of *HSP104* in strains expressing a 2:1 wildtype-to-Q24R ratio or a 1:1 wildtype-to-G58D ratio led to the accumulation of both fast and slowly migrating aggregates (Fig. 8b and Fig. 9b), suggesting that Hsp104 activity was only partially limiting in these strains. Consistent with that idea, the heterozygous

disruption of *HSP104* only partially suppressed PNM effects on the $[PSI^+]$ phenotype in these strains (Fig. 2a) and suppressed $[PSI^+]$ loss in the G58D but not the Q24R-expressing strain (Fig. 2b). In a strain expressing a 1:1 wildtype-to-Q24R ratio, disruption of one copy of *HSP104* failed to alter the migration of Q24R-containing aggregates (Fig. 8b and Fig. 9c), suggesting that Hsp104 activity remained in excess under these conditions, and, correspondingly, prion propagation remained inhibited (Fig. 2a, b). Together, these correlations strongly suggest that Hsp104-mediated fragmentation is a crucial event in the dominant inhibition of prion propagation by PNM mutants *in vivo*.

If this hypothesis is correct, prion propagation in PNM-expressing strains should be hypersensitive to excess Hsp104. To test this idea, we first determined the ratio of Sup35 and Hsp104 expression levels in $[PSI^+]$ strains derived from two genetic backgrounds, 74-D694 and BSC783/4c, which differ in their sensitivities to G58D expression (Doel et al. 1994; Derkatch et al. 1999). In the more sensitive BSC783/4c strain, Hsp104 expression is elevated by a factor of ~2.5 relative to Sup35 in comparison with 74-D694 (Fig. 8c), and the expression levels of the Hsp104 co-chaperones Ssa1 and Sis1 are also elevated in BSC783/4c (Fig. 10a, b). Correspondingly, aggregates in BSC783/4c were shifted to faster migrating complexes by SDD-AGE (Fig. 10c), consistent with the idea that increased sensitivity to PNM inhibition correlates with elevated fragmentation activity. To rule out the possibility that this hypersensitivity resulted from other genetic differences between the two strains, we over-expressed Hsp104 to various levels (Fig. 8d) in 74-D694 wildtype haploid strains that co-expressed

either wildtype or PNM Sup35 (Fig. 4a, b) and determined the frequency of $[PSI^+]$ loss. In a strain expressing wildtype Sup35, $[PSI^+]$ loss was only observed when Hsp104 was overexpressed by factor of ~ 20 ($2\mu_{PGPD}$, Fig. 8d and Fig. 7d). In contrast, $[PSI^+]$ loss was observed in both G58D and Q24R expressing strains with only a single extra copy of Hsp104 (cen_{P104}), and these levels were elevated further upon overexpression of Hsp104 by a factor of ~ 5 and ~ 20 ($2\mu_{P104}$ and $2\mu_{PGPD}$, Fig. 8d and Fig. 7d). Thus, increasing Hsp104 activity can elevate the sensitivity of a $[PSI^+]$ strain to curing by PNM mutants.

Based on this link between the strength of PNM dominant inhibition and Hsp104 activity, we hypothesized that PNM expression induces $[PSI^+]$ curing by promoting the disassembly of aggregates *in vivo*. To monitor the fate of aggregated Sup35, we inhibited new protein synthesis by cycloheximide treatment and then determined the physical state of the existing protein at a later time point. In lysates prepared from a diploid strain expressing two wildtype copies of *SUP35*, the proportion of SDS-resistant Sup35 remained the same, indicating that previously aggregated protein remained in this fraction (Fig. 8e). In contrast, $\sim 50\%$ of the previously aggregated protein, isolated from a strain expressing both wildtype and either G58D or Q24R Sup35s, was resolubilized (Fig. 8e), indicating that incorporation of PNM mutants promotes aggregate disassembly *in vivo*.

Because G58D and Q24R mediate their dominant-negative effects on $[PSI^+]$ propagation by targeting distinct events, we reasoned that combining these mutations into a single polypeptide would create an even more potent inhibitor.

Indeed, the colony color of a $[PSI^+]$ haploid strain expressing a Q24R/G58D double mutant was red in contrast with the pink color of colonies formed from strains expressing either single mutant (Fig. 4a, b). Moreover, $[PSI^+]$ was lost during mitotic division in a strain expressing Q24R/G58D at a greatly enhanced frequency relative to strains expressing either the Q24R (by a factor of ~6) or G58D (by a factor of ~85) single mutants (Table 1). Thus, endogenous quality control pathways can effectively compete with the process of $[PSI^+]$ propagation if mild inhibitors targeting different events in the Sup35 misfolding pathway are combined.

Discussion

Our studies indicate that the QNR and OPR regions make equally important but functionally distinct contributions to the efficient autocatalytic misfolding of Sup35 *in vivo* and, therefore, to the stability of the associated phenotype. Based on our analyses of the effects of Q24R and G58D expression, the QNR region is crucial for conversion to the prion form, while the OPR region determines the stability of aggregates and thereby their frangibility *in vivo*. Our observations are consistent with previous structural and genetic analyses of Sup35 (DePace et al. 1998; Santoso et al. 2000; Osherovich et al. 2004; Krishnan and Lindquist 2005; Tessier and Lindquist 2007; Toyama et al. 2007). However, the PNM-induced alterations are mild (i.e. changed by a factor of ~2), suggesting that their dominant-negative effects cannot be explained simply by the failure to replicate the misfolded species. Rather, these mutations exert their effects on a cellular level by promoting the chaperone-mediated disassembly of aggregates through

different pathways: by lowering the substrate-to-enzyme ratio in the case of Q24R or by enhancing fragmentation efficiency in the case of G58D. While over-expression of Hsp104 was originally thought to induce aggregate disassembly, mounting biochemical and genetic evidence suggests that the excessive level of Hsp104 required to cure wildtype $[PSI^+]$ strains is likely to act through an unknown pathway rather than through an elevated rate of fragmentation (Kryndushkin et al. 2003; Moosavi et al. 2010; Reidy and Masison 2010). Thus, our studies are the first to demonstrate that existing aggregates can be directly and efficiently resolved by endogenous chaperones.

By uncovering the mechanism of prion curing by G58D expression, we have resolved existing conundrums in the field. First, the discrepancy in G58D effectiveness according to genetic background (Doel et al. 1994; Derkatch et al. 1999) can be explained by differences in levels of Hsp104, Hsp70, and Hsp40. Second, the dominant-negative effects of G58D expression had been previously reported to be conformation-specific: while G58D expression cured the Sup35 “strong” conformation ($[PSI^+]^{Strong}$), it stabilized propagation of the “weak” conformation ($[PSI^+]^{Weak}$), which is normally associated with ~3% loss (Derkatch et al. 1999). $[PSI^+]^{Weak}$ and $[PSI^+]^{Strong}$ aggregates differ in their self-replication rates, with $[PSI^+]^{Weak}$ directing more efficient conversion and less efficient fragmentation than $[PSI^+]^{Strong}$ (Tanaka et al. 2006). While the changes induced by G58D are clearly incompatible with $[PSI^+]^{Strong}$, these same changes specifically counteract both of the defects of $[PSI^+]^{Weak}$, providing a molecular explanation for the conformation-specific effects (Derkatch et al. 1999).

Intriguingly, the correlation between effective inhibitory ratio and mechanism of interference for the PNM mutants may also hold for at least two dominant-negative PrP mutants. *In vitro*, the dominant-negative hamster PrP Q172R mutant appears to function analogously to Sup35 Q24R, converting to the prion form inefficiently and inhibiting conformational self-replication at a substoichiometric level (Bossers et al. 1997; Geoghegan et al. 2009). In contrast, the dominant-negative hamster PrP Q219K mutant behaves with strikingly similarity to Sup35 G58D. PrP Q219K is efficiently incorporated into aggregates, (Lee et al. 2007; Geoghegan et al. 2009; Hizume et al. 2009) but this incorporation alters the physical properties of these complexes, lowering their sensitivity to proteolysis (Lee et al. 2007). Consistent with the behavior of Sup35 G58D, PrP Q219K effectively inhibits prion propagation only when present in excess to wildtype PrP *in vitro*, (Geoghegan et al. 2009) implying that this mutant impacts the frangibility of aggregates *in vivo*. Indeed, Q219K, like G58D, exhibits conformation-specific inhibition of prion propagation *in vivo*, acting as a dominant negative toward the less thermodynamically stable Ch/RML variant but not the more thermodynamically stable 22L variant (Safar et al. 1998; Atarashi et al. 2006). These remarkable parallels between the two systems suggest that the pathways mediating prion curing by PNM mutants in yeast may provide a new framework for revealing mechanistic insight into the dominant inhibition of prion propagation in mammals.

Together, our studies suggest that the interplay between the sequence, conformation and cellular environment of a prion protein combine to determine

the balance between the assembly and disassembly of aggregates and thereby the stability of the associated phenotypes. Thus, it is not simply the physical attributes of aggregates that normally preclude their resolution but rather their assembly pathway, which is too efficient to allow on-going disassembly events to effectively compete. Our observations indicate that this balance can be reversed by mild disruption of either the conversion or the fragmentation step of the misfolding pathway *in vivo*. While attempts to cure established prion infections with externally delivered dominant-negative PrP mutants have yet to succeed in animals, (Crozet et al. 2004; Furuya et al. 2006; Toupet et al. 2008) our observations of prion curing in the yeast system indicate that the combination of functionally distinct mutations or the expression of dominant-negative mutants in cells with altered chaperone levels lead to the potent inhibition of prion propagation *in vivo*. Thus, a system-based approach to prion intervention represents a potentially promising direction in which to explore future therapies.

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Author Contributions

SD and TRS designed the experiments, analyzed the data and wrote the manuscript, and SD, AD, and JAP performed the experiments.

Table 1: Spontaneous frequencies of [*PSI*⁺] loss during mitotic division

Strain	[<i>PSI</i> ⁺] Loss (% [<i>psi</i> ⁻])	Colonies Scored
SY1511 (WT+WT)	0	2048
SY1512 (WT+G58D)	0.35	3985
SY1513 (WT+Q24R)	5.13	3239
SY1878 (WT+Q24R/G58D)	29.76	1952

Table 2: Plasmids

Name	Description
6686	pRS306-P ₃₅ Sup35
SB518	pRS305-P ₃₅ Sup35
SB467	pRS306-P ₃₅ Sup35(G58D)
SB410	pRS306-P ₃₅ Sup35(Q24R)
SB590	pRS426-P ₁₀₄ Hsp104
SB629	pRS305-P ₃₅ Sup35-HA
SB641	pRS304-Sup35
SB642	pRS304-Sup35(G58D)
SB643	pRS304-Sup35(Q24R)
SB644	pRS304P ₃₅ Sup35
SB645	pRS304P ₃₅ Sup35(G58D)
SB646	pRS304P ₃₅ Sup35(Q24R)
SB657	pRS306-P _{tetO2} Sup35
SB658	pRS306-P _{tetO2} Sup35(G58D)
SB659	pRS306-P _{tetO2} Sup35(Q24R)
SB675	pRS305-P ₃₅ G58D-HA
SB679	pRS305-P ₃₅ Q24R-HA
SB683	pRS306-P _{tetO2} Sup35(Q24K)
SB685	pRS306-P _{tetO2} Sup35(Q24N)
SB687	pRS306-P _{tetO2} Sup35(Q24A)
SB733	pRS306-P _{tetO2} Sup35(Q24R, G58D)

Table 3: Oligonucleotide Sequences

Name	Sequence
TRS89	5' CGGGATCCCTCGAGAAGATATCCATC 3'
TRS123	5' GGAATTCTGTTGCTAGTGGGCAGA 3'
TRS17	5' CGGGATCCATGTCGGATTCAA 3'
TRS35	5'GTGGAGCTCTTACTCGGCAATTTTAACAATTTTAC 3'
SD25	5' CTGGGTACCAACAAGATGGCTATCAACAG 3'
SD26	5' CTGTTGATAGCCATCTTGTGGTACCCAG 3'
SD1	5' CAGAACGGTAACCAACAAAGAGGTAACAACAGATACCAA 3'
SD2	5' TTGGTATCTGTTGTTACCTCTTTGTTGGTTACCGTTCTG 3'
SD29	5' CAGAACGGTAACCAACAAGCAGGTAACAACAGATACCAA 3'
SD30	5' TTGGTATCTGTTGTTACCTGCTTGTGGTTACCGTTCTG 3'
SD31	5' CAGAACGGTAACCAACAAAATGGTAACAACAGATACCAA 3'
SD32	5' TTGGTATCTGTTGTTACCATTTTGTGGTTACCGTTCTG 3'
SD33	5' CAGAACGGTAACCAACAAAAAGGTAACAACAGATACCAA 3'
SD34	5' TTGGTATCTGTTGTTACCTTTTTGTGGTTACCGTTCTG 3'
SD27	5' ACTTGCTCGGAATAACATCTATATCTGCCCACTAGCAACAC AGCTGAAGCTTCGTACGC 3'
SD28	5' GGTATTATTGTGTTTGCATTTACTTATGTTTGCAAGAAATGC ATAGGCCACTAGTGGATCTG 3'
TRS218	5' GGTAGTTTTGCTGATTGTTGCCTTGGTTTGAATCCGACATAT AGGCCACTAGTGGATCTG 3'
TRS219	5' CTCGAGAAGATATCCATCATATTACCATTGTAATACTGCCCA GCTGAAGCTTCGTACGC 3'

Table 4: Yeast Strains

Strain	Genotype	Plasmids Integrated	Described in figure	Reference
74-D694	<i>MATa</i> (or <i>MATα</i> or <i>MATa/α</i>) [<i>PSI⁺</i>] (or [<i>psi⁻</i>]) <i>ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112</i>	-		Chernoff et al. 1995
SY80	<i>MATα</i> [<i>PSI⁺</i>] <i>ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3, 112 SUP35GFP</i>	-	8,10	Satpute-Krishnan & Serio, 2005
SY1060	<i>MATa/α</i> [<i>PSI⁺</i>] <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112</i>	6686	5,6	This study
SY1085	<i>MATa/α</i> [<i>PSI⁺</i>] <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112</i>	SB410	6	This study
SY1228	<i>MATα</i> [<i>psi⁻</i>] <i>ade1-14 his3Δ200 trp1-289::TRP1::P_{GPD}GST-UGA-DSRED-NLS ura3-52::URA3::P₃₅SUP35 leu2-3,112 sup35::KANMX4</i>	SB531 6686	1, 2, 7c, 8b	This study
SY1229	<i>MATα</i> [<i>psi⁻</i>] <i>ade1-14 his3Δ200 trp1-289::TRP1::P_{GPD}GST-UGA-DSRED-NLS ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112 sup35::KANMX4</i>	SB531 SB467	3b	This study
SY1230	<i>MATα</i> [<i>psi⁻</i>] <i>ade1-14 his3Δ200 trp1-289::TRP1::P_{GPD}GST-UGA-DSRED-NLS ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112 sup35::KANMX4</i>	SB531 SB410	3b	This study
SY1252	<i>MATa/α</i> [<i>PSI⁺</i>] <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	6686	3b	This study
SY1254	<i>MATa/α</i> [<i>PSI⁺</i>] <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200</i>	SB410	1, 2, 7c, 8b	This study

	<i>trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>			
SY1257	<i>MATa/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/trp1-289::TRP1::P₃₅SUP35(G58D) ura3-52/ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB467 SB645	1, 2, 7c	This study
SY1258	<i>MATb/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/trp1-289::TRP1::P₃₅SUP35(Q24R) ura3-52/ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB410 SB646	1, 2, 7c	This study
SY1359	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112</i>	SB467	1, 2, 7c, 8b	This study
SY1360	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	6686	1, 2, 7c, 8b	This study
SY1511	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35 leu2-3,112</i>	SB657	3d, 4a, 5a,b	This study
SY1512	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3,112</i>	SB658	3d, 4a, 5a,b	This study
SY1513	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24R) leu2-3,112</i>	SB659	3d, 4a, 5a,b	This study
SY1577	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::ura3::KANMX4::P_{tetO2}SUP35 leu2-3,112</i>	SB657	8d	This study
SY1578	<i>MATa [PSI⁺] ade1-14 trp1-289 his3Δ200 ura3-52::ura3::KANMX4::P_{tetO2}SUP35(G58D) leu2-3, 112</i>	SB658	8d	This study
SY1579	<i>MATa [PSI⁺] ade1-14 trp1-289 his3Δ200 ura3-52::ura3::KANMX4::P_{tetO2}SUP35(Q24R) leu2-3, 112</i>	SB659	8d	This study

SY1580	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35 leu2-3, 112 SUP35GFP</i>	SB657	5c	This study
SY1581	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3, 112 SUP35GFP</i>	SB658	5c	This study
SY1603	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112::LEU2::P₃₅SUP35-HA</i>	SB629	3a	This study
SY1604	<i>MATα [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112::LEU2::P₃₅SUP35-HA</i>	SB629	3a	This study
SY1605	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112::LEU2::P₃₅SUP35(G58D)-HA</i>	SB675	3a	This study
SY1606	<i>MATα [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112::LEU2::P₃₅SUP35(G58D)-HA</i>	SB675	3a	This study
SY1622	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112::LEU2::P₃₅SUP35(Q24R)-HA</i>	SB659	3a	This study
SY1623	<i>MATα [psi⁻] ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112::LEU2::P₃₅SUP35(G58D)-HA</i>	SB659	3a	This study
SY1630	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24N) leu2-3,112</i>	SB685	4b-d	This study
SY1631	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24K) leu2-3,112</i>	SB683	4b-d	This study
SY1634	<i>MATα [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24A) leu2-3,112</i>	SB687	4b-d	This study
SY1646	<i>MATα/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35 leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB657	7a, 8a	This study
SY1648	<i>MATα/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB658	7a, 8a	This study

SY1650	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB659	7a, 8a	This study
SY1703	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/his 3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	6686	2	This study
SY1704	<i>MATa/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112 HSP104/hsp104::LEU2</i>	6686	2	This study
SY1705	<i>MATa/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 HSP104/hsp104::LEU2</i>	SB467	2	This study
SY1706	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/his 3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	SB467	2	This study
SY1707	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/his 3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	SB410	2	This study
SY1708	<i>MATa/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3 112/ leu2-3,112 HSP104/hsp104::LEU2</i>	SB410	2	This study
SY1710	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35 leu2-3,112/ leu2-3,112</i>	SB657	7a,b, 8a	This study

	<i>SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>			
SY1711	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	SB658	7a,b, 8a	This study
SY1712	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	SB659	7a,b, 8a	This study
SY1771	<i>MATa/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/3 trp1-289::TRP1::P₃₅SUP35(G58D) ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	SB645 SB467	2	This study
SY1772	<i>MATa/α [PSI⁺] ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/3 trp1-289::TRP1::P₃₅SUP35(Q24R) ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4 HSP104/hsp104::LEU2</i>	SB646 SB410	2	This study
SY1878	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24R/ G58D) leu2-3,112</i>	SB733	4a	This study

Methods

Plasmid Construction

All plasmids generated in this study are listed in Table 2. pRS306-P₃₅Sup35 (6686) and pRS305-P₃₅Sup35 (SB518) contain the Sup35 promoter (P₃₅) as a *Bam*HI-*Eco*RI fragment (TRS89, TRS123) and the Sup35 open reading frame (ORF) as a *Bam*HI-*Sac*I fragment (TRS17, TRS35), generated by PCR using the indicated primers (see Table 3), subcloned into pRS306 and pRS305, respectively. All plasmids encoding Sup35 PNM mutants were created using QuickChange mutagenesis (Stratagene) according to the manufacturer's instructions using 6686 or SB659 as templates and the indicated primers (see Table 3): G58D (SD25, SD26), Q24R (SD1, SD2), Q24K (SD33, SD34) Q24N (SD31, SD32) Q24A (SD29, SD30). pRS306-P_{tetO2}Sup35 (SB657), G58D (SB658), and Q24R (SB659) contain the ORFs as *Bam*HI-*Nae*I fragments isolated from 6686, SB467, and SB410 and subcloned into similarly digested pRS306-P_{CMV}-tTA-tetO2 (a gift from J. Laney). pRS304-Sup35 (SB641), G58D (SB642), and Q24R (SB643) contain the ORFs as *Bam*HI-*Sac*I fragments isolated from pRS306-P₃₅Sup35, SB467, and SB410, respectively, and subcloned to similarly digested pRS304. P₃₅ was then subcloned into these plasmids as a *Bam*HI-*Eco*RI fragment isolated from pRS306-P₃₅Sup35 to create SB644, SB645, and SB646, respectively. pRS305-P₃₅Sup35-HA (SB629) was generated by substituting a *Pst*I-*Sal*I fragment from p316SpSup-HA3-FL (a gift from J. Weissman), which contains a fragment of Sup35 and an (HA)₃ epitope

tag, for the corresponding region of wildtype Sup35. pRS305-P₃₅G58D-HA (SD25, SD26; SB675) and pRS305-P₃₅Q24R-HA (SD1, SD2; SB679) plasmids were generated by QuickChange mutagenesis (Stratagene) according to the manufacturer's instructions using SB629 as a template and the indicated primers. SB590 was constructed by subcloning a *Xho*I-*Sac*I fragment containing the Hsp104 promoter (P₁₀₄) and ORF from pHSE-Hsp104 (a gift from S. Lindquist) into similarly digested pRS426. Plasmids generated by PCR were confirmed by sequencing.

Strain Construction. All yeast strains are derivatives of 74-D694 unless otherwise noted and are listed in Table 4. Yeast strains expressing ectopic copies of *SUP35* or PNM mutants from *URA3* (pRS306), *TRP1* (pRS304), or *LEU2* (pRS305)-based plasmids were constructed by transforming yeast strains with plasmids that were linearized with *Bst*BI, *Bsu*361, or *Afl*II, respectively, and by selecting for transformants on the appropriate minimal media. In all cases, expression was confirmed by quantitative immunoblotting for Sup35. Disruptions of *SUP35* (SD27, SD28) were generated by transformation of PCR-generated cassettes using pFA6aKanMX4 as a template with the indicated primers (see Table 3) and selection on complete media supplemented with G418. *HSP104* disruptions were generated by transformation of *Pvu*I-*Bam*HI fragment of pYABL5 (a gift from S. Lindquist) and selection on minimal media lacking leucine. Disruptions were verified by PCR and 2:2 segregation of the appropriate marker. *URA3* disruptions were generated by transforming M3927 (a gift from D. Stillman) that had been linearized with *Bam*HI and by selection on rich media

supplemented with G418 (300µg/ml). Disruption of *URA3* was confirmed by auxotrophic phenotype. P_{35} was replaced by P_{tetO2} by transformation of PCR-generated cassettes using pCM224 (Belli et al. 1998) as a template and primers (TRS218 and TRS219) and selection on complete media supplemented with G418 (300µg/ml). Replacement was confirmed by PCR, 2:2 segregation of G418 resistance, and doxycycline-induced lethality. Strains expressing GST-UGA-DsRED-NLS were constructed by transformation of *Bsu*361-digested SB531 (Pezza et al. 2009) and selection on minimal media lacking tryptophan.

Imaging and Fluorescence Loss In Photobleaching (FLIP). Experiments were performed on a Zeiss LSM510-META laser scanning confocal microscope equipped with a 63x objective (NA=1.4) and an Argon/Helium Neon laser. Frames (2x zoom, 100 x 100 pixels, 1 Airy unit) were collected using 488-nm excitation and 500-560nm emission wavelengths for GFP-tagged proteins and 516-nm excitation and 575-615-nm emission wavelengths for DsRED-tagged proteins. Studies analyzing GST-UGA-DsRED-NLS fluorescence in zygotes were performed as previously described. (Satpute-Krishnan and Serio 2005) To quantify fluorescence intensities, nuclei from individual cells were analyzed for mean pixel intensity using ImageJ (NIH) image analysis software, and data were normalized to the wildtype mean. For FLIP, laser intensity was set to 5% for image acquisition and 100% for bleaching for 8 iterations. Cells with buds ~1/3 their size were chosen for analysis, and average fluorescence pixel intensity was monitored in the mother cell, the bud, and a nearby control cell, with bleaching of the entire bud following each image acquisition. Image analysis was performed

using Zeiss LSM Image Analysis software, where specific values were normalized to background levels of fluorescence loss and presented as time post-initial bleach.

Protein Analysis. Semi-denaturing agarose gel electrophoresis, SDS-PAGE, and quantitative immunoblotting, SDS-sensitivity were performed as previously described.(Pezza et al. 2009) Anti-Ssa1 rabbit serum was provided by E. Craig, and anti-Sis1 rabbit serum was provided by M. Tuite. Immunoprecipitations were performed as previously described,(Bagriantsev et al. 2008) with the following modifications. Cells were harvested in early log phase; the lysis buffer was supplemented with $5\mu\text{g ml}^{-1}$ pepstatin, Complete protease inhibitor tablet (Roche Applied Sciences), $4\mu\text{l ml}^{-1}$ Protease Inhibitor Cocktail (Sigma), and 1 mg ml^{-1} BSA, and anti-HA high affinity rat monoclonal clone 3F10 (Roche Applied Sciences) was used.

Propagon Counts. The number of propagons per cell was determined by an *in vivo* dilution, colony-based method, as previously described.(Cox et al. 2003)

Curing by Hsp104 Overexpression. Yeast strains were transformed with vector, pHSE-Hsp104 (a gift from S. Lindquist), SB590 or pLH102 (a gift from S. Lindquist) and plated on minimal medium lacking uracil. Individual colonies were transferred to YPD plates, incubated at 30°C overnight, and transferred to 5-FOA plates. Individual colonies were then isolated on $\frac{1}{4}$ YPD agarose plates. Curing was scored by the appearance of red colonies.

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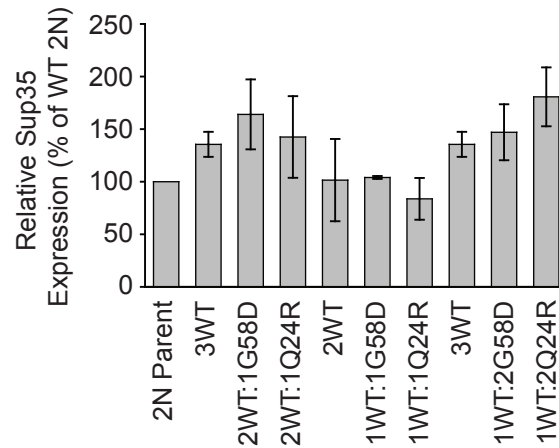


Figure 1. Sup35 expression level is consistent with copy number. Lysates isolated from wildtype (HSP104/HSP104) or heterozygous disruption (HSP104/ Δ) diploid strains expressing wildtype (WT) and PNM mutants (Q24R, G58D) from P35 at the indicated ratios were analyzed by SDS-PAGE and quantitative immunoblotting for Sup35. A wildtype diploid (2N Parent) is included as a control. Expression levels are consistent with Sup35 copy number. Error bars represent standard deviation ($n \geq 3$).

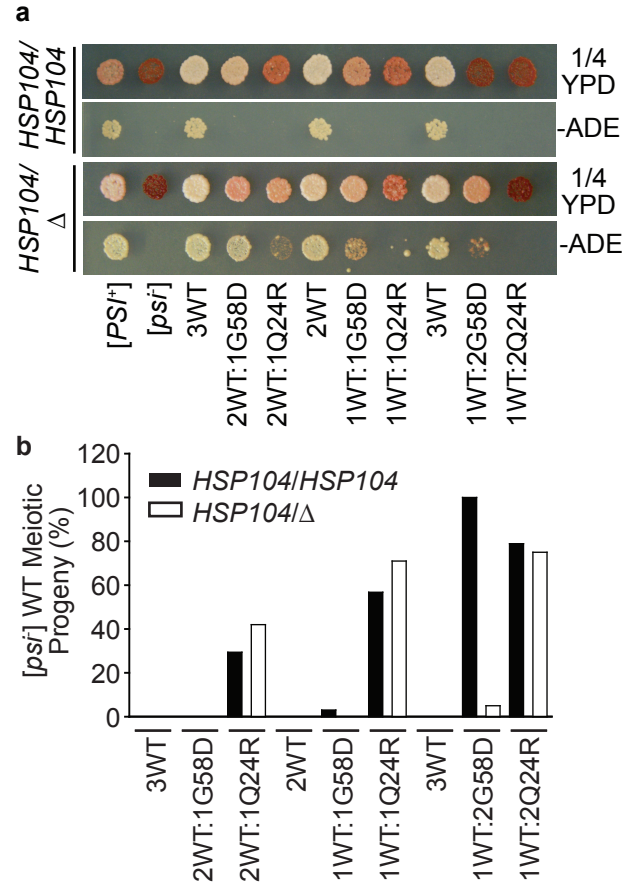


Figure 2. PNM mutants are distinguished by their effective inhibitory ratios. a. Wildtype (HSP104/HSP104) or heterozygous disruption (HSP104/Δ) diploid strains expressing wildtype (WT) and PNM mutants (Q24R, G58D) from P35 at the indicated ratios were spotted on rich (1/4 YPD) or adenine deficient (-ADE) media to analyze the *[PSI⁺]* phenotype. Wildtype *[PSI⁺]* and *[psi⁻]* diploids (74-D694) were included as controls. b. To determine the frequency of prion loss, wildtype meiotic progeny ($n \geq 19$ for each strain) were isolated from the diploids described in (a), and the percentage of *[psi⁻]* colonies was determined.

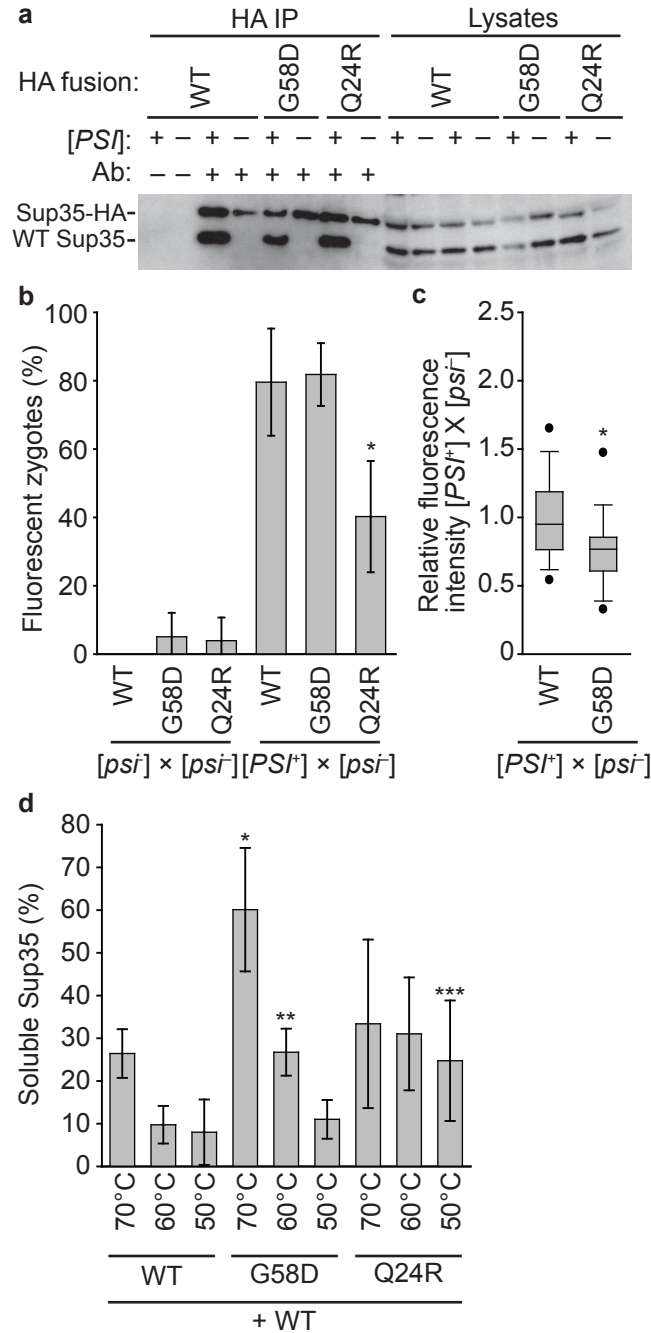


Figure 3. PNM mutants incorporate into wildtype aggregates and alter multiple events in prion propagation. **a.** HA-tagged Sup35 (WT or mutants) expressed from P_{35} in haploid $[PSI^+]$ (+) or $[psi^-]$ (–) yeast strains, which also expressed untagged Sup35, was immunoprecipitated with anti-HA serum (Ab) and analyzed by SDS-PAGE and anti-Sup35 immunoblotting. **b.** $[psi^-]$ haploids expressing Sup35 (WT or mutants) from P_{35} and a fluorescent reporter of translation termination efficiency (GST-UGA-DsRed-NLS) were mated to wildtype $[PSI^+]$ or $[psi^-]$ (74-D694) cells, and the percentage of fluorescent zygotes was scored. Error

bars represent standard deviation from three independent experiments, each analyzing at least 15 zygotes per cross (* $p=0.039$ in comparison with WT). c. The fluorescence intensities of zygotes isolated from the indicated crosses as described in (b) were determined. Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles; error bars indicate 10th and 90th percentiles, and dots represent outliers ($n \geq 30$; * $p=0.0009$). d. Lysates from wildtype haploid strains expressing an additional copy of Sup35 (WT or mutants) from P_{tetO2} were incubated in SDS at the indicated temperatures before SDS-PAGE and quantitative immunoblotting for Sup35 (percentage of Sup35 at the indicated temperatures relative to 100°C). Error bars represent standard deviation ($n \geq 6$, * $p = 0.0003$, ** $p = 0.0001$, *** $p=0.008$ in comparison with WT at the same temperature).

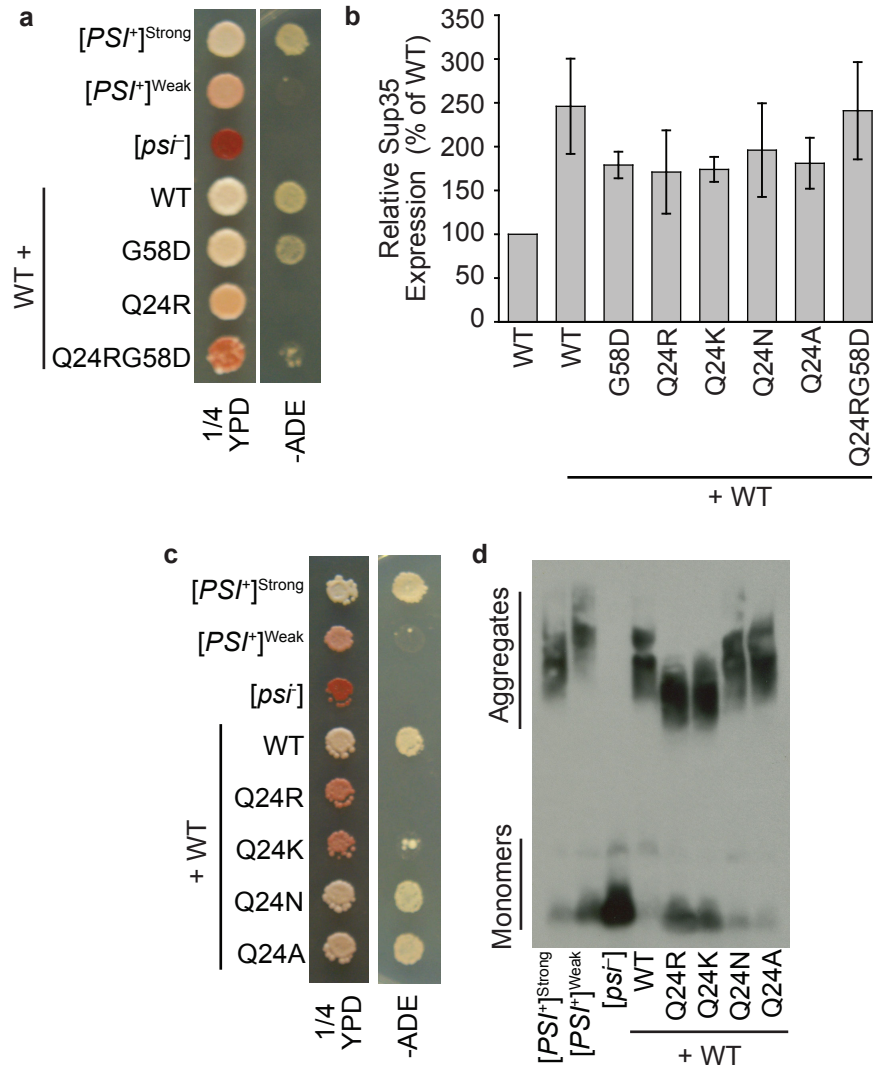


Figure 4. Effects of PNM mutants on prion propagation *in vivo*. **a.** Haploid strains expressing a second copy of Sup35 (wildtype or mutants) from P_{tetO2} were spotted on rich (1/4 YPD) or adenine deficient (-ADE) media to analyze translation termination efficiency. Wildtype [PSI⁺]^{Strong}, [PSI⁺]^{Weak}, and [psi⁻] haploid strains are shown as controls. **b.** Lysates isolated from the indicated strains were analyzed by SDS-PAGE and quantitative immunoblotting for Sup35. Expression levels are consistent with Sup35 copy number. A wildtype [PSI⁺]^{Strong} haploid strain is shown as a control. Error bars represent standard deviation ($n \geq 3$). **c.** Haploid strains expressing a second copy of Sup35 or mutants) from P_{tetO2} were spotted on rich (1/4 YPD) or adenine deficient (-ADE) media to analyze translation termination efficiency. Wildtype [PSI⁺]^{Strong}, [PSI⁺]^{Weak}, and [psi⁻] haploid strains are shown as controls. **d.** Lysates isolated from the strains described in (c) were analyzed by SDD-AGE and immunoblotting for Sup35.

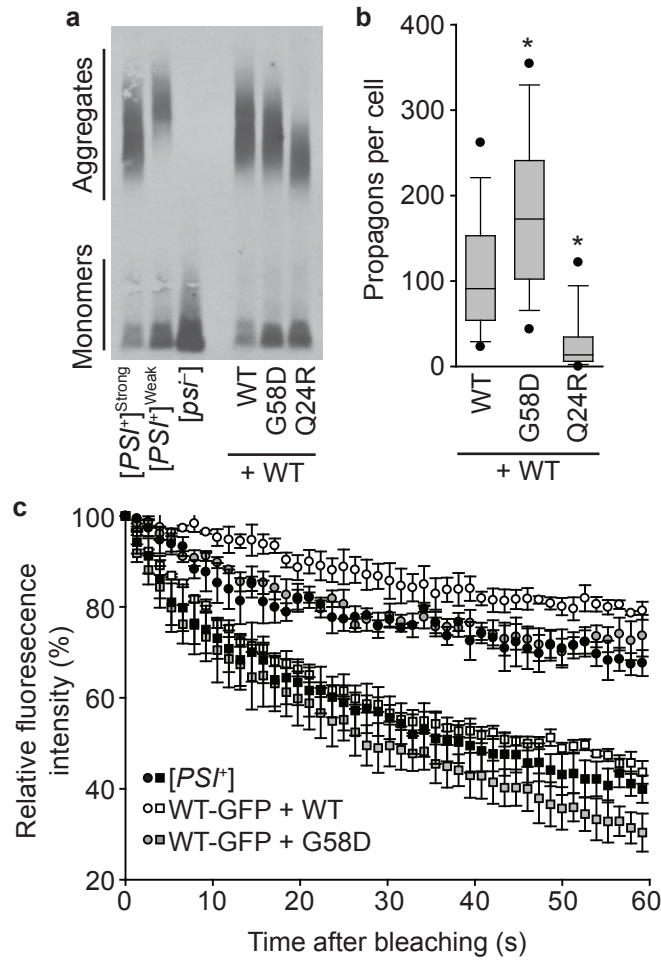


Figure 5. PNM mutants alter the accumulation of propagons but not their transmission. **a.** Lysates from haploid wildtype yeast strains expressing Sup35 (WT or mutants) from P_{tetO2} were analyzed by SDD-AGE and immunoblotting for Sup35. Wildtype [PSI⁺]^{Strong}, [PSI⁺]^{Weak}, and [psi⁻] yeast strains are shown as controls. **b.** The number of propagons present in individual cells was determined for the indicated strains, as described in (a). Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles; error bars indicate 10th and 90th percentiles, and dots represent outliers ($n \geq 39$; * $p \leq 0.0001$ in comparison with WT). **c.** The proportion of Sup35 transmitted to daughter cells (circles) or to mother cells (squares) was determined by fluorescence loss in photobleaching (FLIP) of a [PSI⁺] strain expressing Sup35-GFP alone ([PSI⁺]) or with a second copy of Sup35 (WT-GFP + WT) or G58D (WT-GFP + G58D) from P_{tetO2} . Error bars represent standard error of the mean from three independent experiments, each analyzing at least 10 cells.

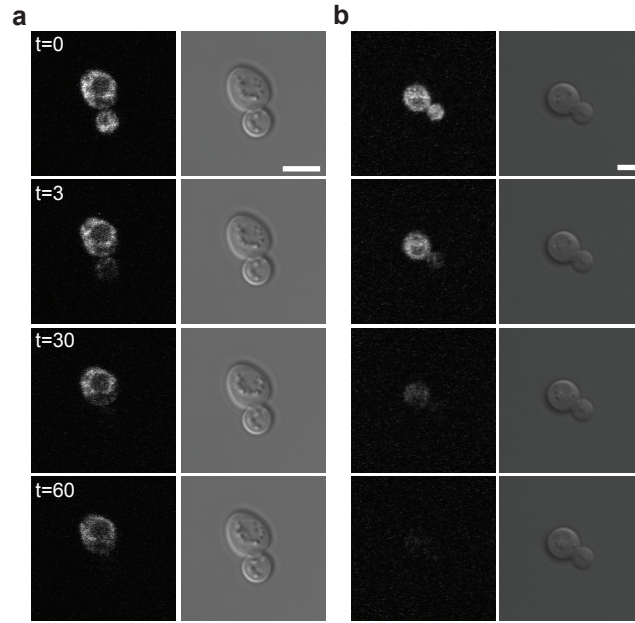


Figure 6. FLIP analysis of Sup35-GFP transmission. Fluorescent (left) and bright-field (right) images of **a.** [*PSI*⁺] or **b.** [*psi*] cells expressing Sup35-GFP are shown pre-bleach (t=0) or over a time course during repeated bleaching of the daughter cell. Time (t) was recorded in seconds. Scale bars represent 5μm.

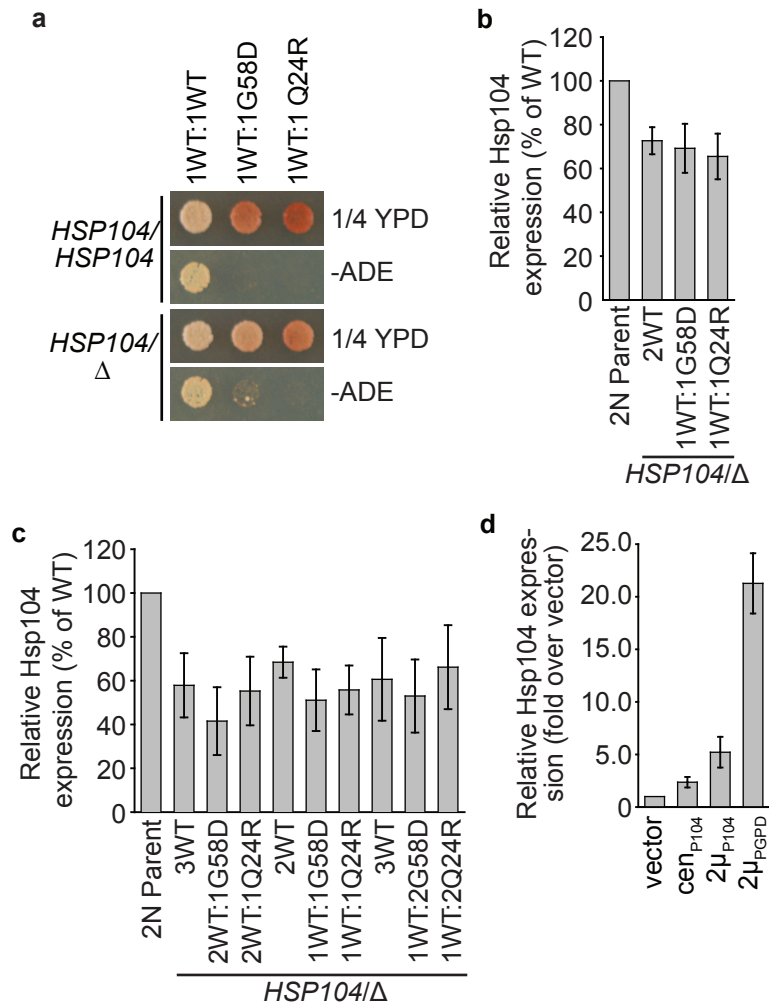


Figure 7. Changes in Hsp104 expression alter PNM effects. **a.** Diploid strains expressing one endogenous copy of Sup35 (WT) and one copy of Sup35 (wildtype or mutants) from P_{tetO2} in a wildtype *HSP104* background (*HSP104/HSP104*) or in an *HSP104* heterozygous disruption background (*HSP104/Δ*) were spotted on rich (1/4 YPD) or adenine deficient (-ADE) media to analyze translation termination efficiency. **b.** Lysates from diploid strains containing a heterozygous disruption of *HSP104* (*HSP104/Δ*) and the indicated ratios of Sup35 (WT) and mutants expressed from **b.** P_{tetO2} or **c.** P_{35} were analyzed by SDS-PAGE and quantitative immunoblotting for Hsp104. Error bars represent standard deviation ($n \geq 3$). Expression levels are consistent with copy number. **d.** Lysates from haploid [*PSI*⁺] strains transformed with the indicated Hsp104 expression plasmid or vector control were analyzed by SDS-PAGE and quantitative immunoblotting for Hsp104. Error bars represent standard deviation ($n \geq 3$).

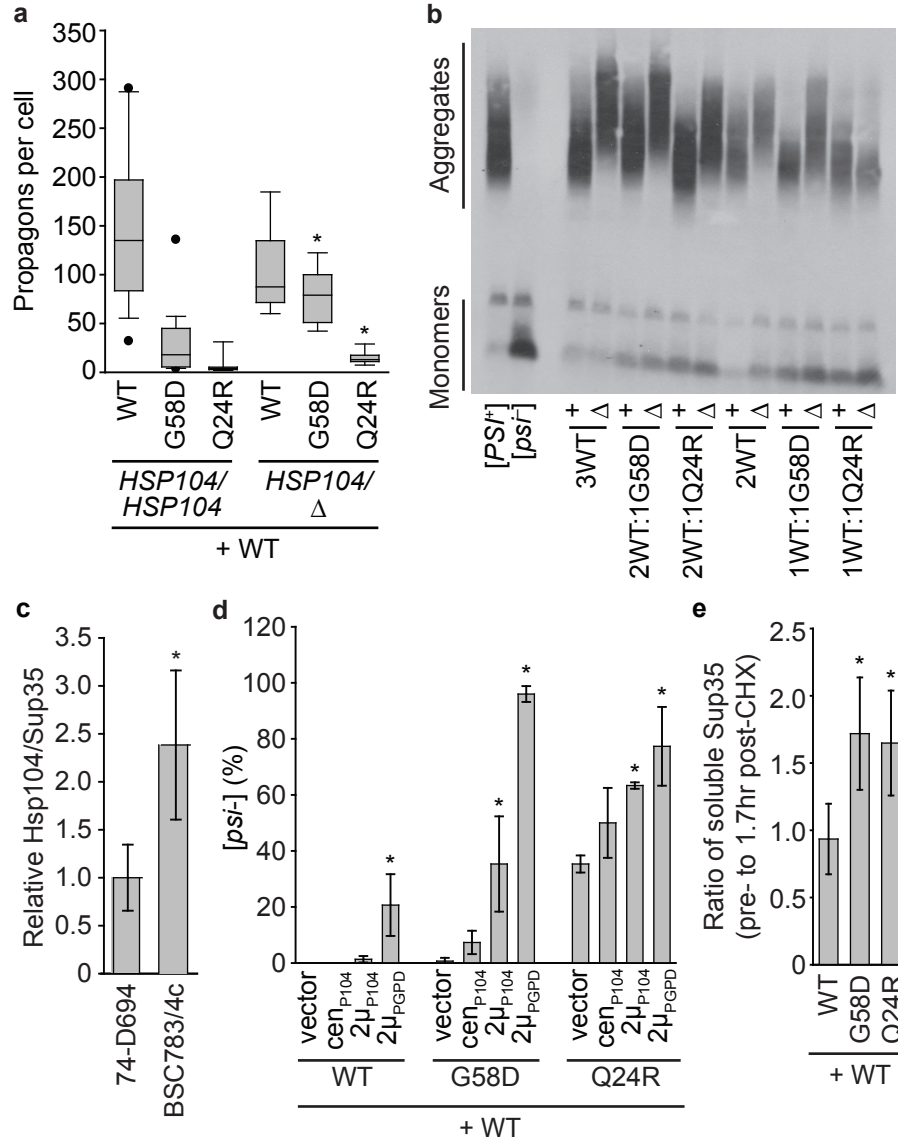


Figure 8. PNM expression promotes Hsp104-mediated disassembly of aggregates. a. The number of propagons present in individual cells was determined for diploid [*PSI*⁺] strains expressing one endogenous copy of SUP35 and one copy of SUP35 (WT or mutants) from *P*_{tetO2} in a wildtype background (HSP104/ HSP104) or in a heterozygous disruption background (HSP104/Δ). Box plots are as described in the legend to Fig. 3b. *n*≥10 cells per strain; **p* < 0.05, in comparison with the corresponding HSP104/HSP104 strain. b. Lysates of diploid strains expressing Sup35 (WT) and PNM mutants in the indicated ratios were analyzed by SDD-AGE and immunoblotting for Sup35. Wildtype (+) and heterozygous disruption of HSP104 (Δ) are indicated. c. Lysates from BSC783/4c and 74-D694 [*PSI*⁺] haploids were analyzed by SDS-PAGE and quantitative immunoblotting for Hsp104 and Sup35. Error bars represent standard deviation (*n*=5, **p*=0.0031). d. Haploid [*PSI*⁺] cells co-expressing endogenous SUP35 and a second copy of

SUP35 (WT or mutants) from P_{tetO2} were transformed with pHSE-Hsp104 (cenP104), SB590 (2 μ P104), pLH102 (2 μ PGPD) or a vector control, and the percentage of [*psi*⁻] colonies was scored after plasmid loss. Error bars represent standard deviation from three independent experiments, each analyzing a total of 50 colonies (* $p < 0.05$, in comparison with vector control for the same strain). e. Lysates from diploid [*PSI*⁺] strains expressing two copies of SUP35 (WT) or one wildtype and one mutant copy of SUP35 from P_{tetO2} were incubated in SDS at 50°C or 100°C before SDS-PAGE and quantitative immunoblotting for Sup35, and the ratio of signal before and after cycloheximide treatment was determined. Error bars represent standard deviation ($n \geq 3$, * $p = 0.029$, ** $p = 0.011$).

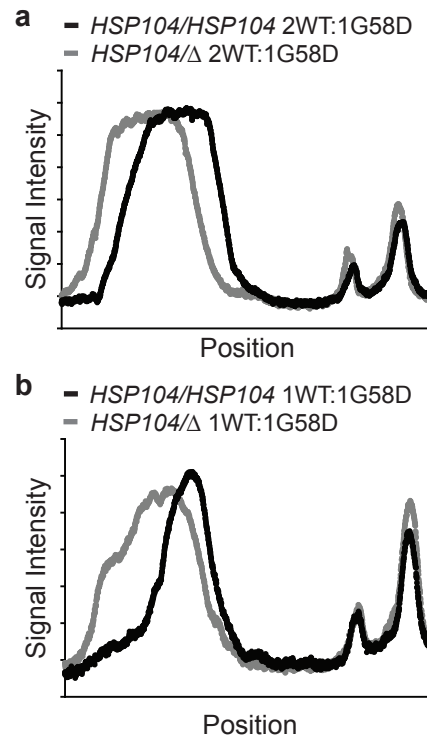


Figure 9. Densitometry traces. Comparisons of the indicated lanes from the agarose gel shown in Figure 4c were generated with ImageJ.

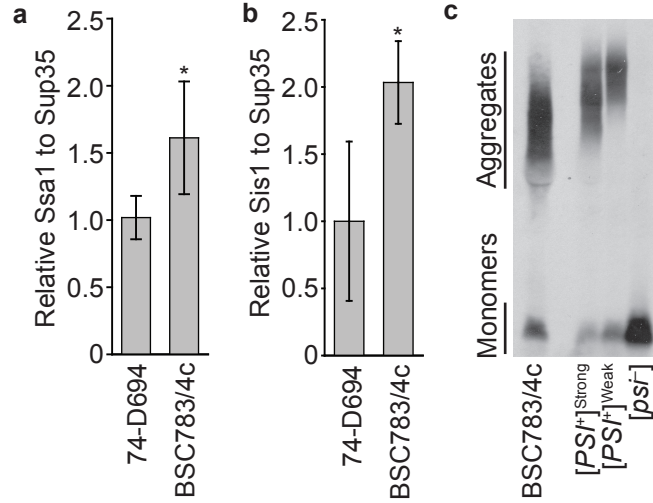


Figure 10. Fragmentation activity is dependent on yeast genetic background. Lysates from 74-D694 and BSC783/4c [*PSI*⁺] haploids were analyzed by SDS-PAGE and quantitative immunoblotting for **a**. Ssa1 (Hsp70) and Sup35 or **b**. Sis1 (Hsp40) and Sup35. Error bars represent standard deviation (n≥3, *p=0.018 and *p=0.036, respectively). **c**. Lysates prepared from BSC783/4c [*PSI*⁺], 74-D694 [*PSI*⁺]^{Strong} or [*PSI*⁺]^{Weak}, and 74-D694 [*psi*⁻] strains were analyzed by SDD-AGE and immunoblotting for Sup35.

**Chapter 3: The Relationship between Prion Sequence Mutants and the
Prion Conformer: Prion Mutants Differentially Affect the Phenotypic
Outcome of Distinct Prion Variants**

I performed all the experiments described in this section

Abstract

Prions are self-replicating transmissible protein aggregates that can give rise to unique phenotypes. Interestingly, despite being conferred by aggregates composed of identical protein, several distinguishable phenotypes, or variants, can be associated with each prion. Alterations in aggregate properties underpin this phenomenon. For the yeast prion $[PSI^+]$ system, a “strong” prion variant displays a more severe prion-associated phenotype than a “weak” prion variant; this effect is mediated by the greater frangibility of the strong aggregate, which leads to more efficient prion amplification and transmission. Prion sequences can also modulate aggregate properties and thereby phenotype, with some prion mutants demonstrating variant-specific inhibition. Here, we examine two Sup35/ $[PSI^+]$ prion mutants, G58D and Q24R, both of which act as dominant-negatives on $[PSI^+]^{\text{Strong}}$, for their effects on the $[PSI^+]^{\text{Weak}}$ prion variant. We found that the phenotypic effect of these mutants is strongly dependent on the underlying prion variant, with G58D and Q24R affecting the $[PSI^+]^{\text{Weak}}$ variant in opposing ways. We suggest that these differential physiological outcomes can be explained by the intersection of sequence and conformational perturbations on prion aggregate physical properties rather than through distinct cellular pathways.

Introduction

Prion proteins act as conformation-based transmissible elements due to their capacity to form multiple stable conformations, some of which self-replicate by converting like proteins into the same state (Griffith 1967; Prusiner 1982). Self-replicating conformers assemble into higher order aggregates, which alter their normal cellular activity and are stably propagated *in vivo* to create heritable phenotypes (Tuite and Serio 2010). Together, the self-replication and transmissibility of these aggregates provide an explanation for the infectivity and progressive neurodegeneration associated with the transmissible spongiform encephalopathies in man and animals and for the non-Mendelian inheritance of several epigenetic traits in fungi (Tuite and Serio 2010). Interestingly however, prion complexes composed of the same protein can confer a spectrum of distinguishable phenotypes (prion variants) that typically differ in severity, an outcome that is modulated by the particular conformation of the prion protein in the aggregate and by its cellular environment (Legname et al. 2006; Tanaka et al. 2006; Pezza and Serio 2007; Derdowski et al. 2010; Ayers et al. 2011). The yeast *Saccharomyces cerevisiae* prion Sup35/[PSI⁺] is well suited for examining how the phenotypic realizations of *in vivo* prion self-replication arise and how perturbations of this process likewise modify the corresponding phenotype.

Sup35 is a translation termination factor that is partially inactivated in its aggregated prion form, leading to a translation termination defect that is easily detected by readthrough of genes encoding premature nonsense mutations. For example, prion ([PSI⁺]) strains carrying the *ade1-14* allele form white colonies on

rich and adenine-deficient media due to readthrough of a premature stop codon in the *ADE1* open reading frame. In contrast, non-prion (*[psi⁻]*) strains form red colonies on rich medium and fail to grow in the absence of adenine due to the accumulation of active, non-prion state Sup35 (Chernoff et al. 1995; Patino et al. 1996; Paushkin et al. 1996). *[PSI⁺]* variants, which do not efficiently sequester the majority of soluble Sup35 in the cell, produce pink colonies on rich medium due to less efficient stop-codon readthrough and are therefore characterized as “weak” variants (Derkatch et al. 1996; Zhou et al. 1999; Tanaka et al. 2006). The *[PSI⁺]^{Strong}* prion state of Sup35 is epigenetically very stable and is infrequently lost during mitosis, but the *[PSI⁺]^{Weak}* prion variant displays slightly elevated instability, producing approximately 0.1-1% *[psi⁻]* colonies during cell division (Derkatch et al. 1996).

This epigenetic stability is underpinned by an *in vivo* aggregate biogenesis cycle in which several critical steps must occur to ensure efficient aggregate transmission to new cells. First, once a prion complex is transmitted or arises in a cell, it must grow through the incorporation of nascent Sup35 monomers, thereby inactivating them (Patino et al. 1996; Satpute-Krishnan and Serio 2005). Next, these complexes must be fragmented by the molecular chaperone Hsp104 to generate more templates for conversion and ensure efficient transmission to daughter cells (Chernoff et al. 1995; Ness et al. 2002; Satpute-Krishnan et al. 2007; Higurashi et al. 2008; Tessarz et al. 2008; Tipton et al. 2008; Kawai-Noma et al. 2009). Furthermore, efficient aggregate transmission to daughter cells is

linked to the number and size range of the replicating prion complexes (Satpute-Krishnan et al. 2007; Kawai-Noma et al. 2009; Derdowski et al. 2010).

Thus, prion variants give rise to distinct phenotypes because the prion conformation specifies the kinetics of aggregate growth and fragmentation, resulting in the accumulation of distinct pools of Sup35 species at steady state (Zhou et al. 1999; Kryndushkin et al. 2003; Bagriantsev et al. 2006; Tanaka et al. 2006). Typically, weak prion conformers are more thermodynamically stable than strong prion conformers, a characteristic that is recapitulated with fibers grown *in vitro* at 37°C (Sc37) and 4°C (Sc4), which respectively produce $[PSI^+]^{\text{Weak}}$ and $[PSI^+]^{\text{Strong}}$ -like phenotypes *in vivo* (Tanaka et al. 2004). The thermodynamic stability of prion variants correlates with their fragmentation efficiencies (Tanaka et al. 2006) and is therefore thought to greatly impact the overall abundance and sizes of prion aggregates within the cell (Tanaka et al. 2006; Derdowski et al. 2010). Thus, $[PSI^+]^{\text{Strong}}$ variants are efficiently amplified, resulting in lower levels of soluble Sup35 and a correspondingly more severe prion phenotype (Tanaka et al. 2006). Moreover, these dynamics create many smaller prion complexes that are more efficiently transmitted during cell division (Kryndushkin et al. 2003; Derdowski et al. 2010). Weak $[PSI^+]$ variants, on the other hand, have higher levels of soluble Sup35 and fewer, larger prion complexes, resulting in a weaker phenotype and less efficient aggregate transmission (Kryndushkin et al. 2003; Tanaka et al. 2006; Derdowski et al. 2010).

In addition to conformational effects, the primary sequence of the prion protein also modulates the phenotypic outcome of prion propagation. The amino acid

composition of prions primarily appears to restrict its spectrum of accessible conformations and thereby distinct phenotypes. Indeed, several prion sequence polymorphisms or mutations are incompatible with specific prion variants, in some cases blocking or disabling their transmission or replication (Young and Cox 1971; Doel et al. 1994; DePace et al. 1998; Lee et al. 2007; Geoghegan et al. 2009). Interestingly however, the same sequence polymorphisms may be compatible with other prion variants (Safar et al. 1998; King 2001; Atarashi et al. 2006; Verges et al. 2011) and, in some situations, actually have the inverse effects of enhancing the transmission or severity of the prion state (Derkatch et al. 1999). For instance, the mammalian PrP mutant Q219K acts as a dominant-negative inhibitor of the Ch/RML variant but not of the 22L variant (Safar et al. 1998; Atarashi et al. 2006). Additionally, the yeast Sup35 mutant G58D dominantly inhibits the $[PSI^+]^{\text{Strong}}$ conformation, but is compatible with several other prion variants (Derkatch et al. 1999; King 2001; Verges et al. 2011). In these latter cases, G58D does not disrupt the prion state when co-expressed with wildtype Sup35 (Derkatch et al. 1999; Verges et al. 2011) and can stably replicate the prion state when present as the only copy of Sup35 (King 2001; Verges et al. 2011). Furthermore, G58D overexpression in a $[PSI^+]^{\text{Weak}}$ strain has been shown to enhance the mitotic stability of this variant, in contrast to the enhanced prion loss caused by wildtype Sup35 overexpression (Derkatch et al. 1999).

In order to better understand the differential phenotypic effects of prion mutants on the $[PSI^+]^{\text{Weak}}$ variant, we studied the molecular effects of Q24R or G58D on

$[PSI^+]^{Weak}$ propagation using the approaches that we had previously developed to assess the effects of these mutants on $[PSI^+]^{Strong}$ propagation (Disalvo et al. 2011). G58D fails to effectively inhibit $[PSI^+]^{Weak}$ prion propagation in any of the conditions examined, consistent with previous reports at one ratio relative to wildtype Sup35 (Derkatch et al. 1999), but expression of this mutant slightly enhanced self-replication of prion state, providing a molecular explanation for the increase in mitotic stability that was previously reported. In contrast, Q24R was a potent inhibitor of the $[PSI^+]^{Weak}$ variant. Surprisingly, co-expression of a double mutant can suppress the effects of Q24R expression, effectively rescuing the $[PSI^+]^{Weak}$ state.

Results

Prion inhibition and effective inhibitory ratios distinguish Sup35 mutants in $[PSI^+]^{Weak}$ strains

Although the dominant-negative effect of G58D and Q24R has previously been examined for $[PSI^+]^{Strong}$ variants (Young and Cox 1971; Doel et al. 1994; DePace et al. 1998; Disalvo et al. 2011; Verges et al. 2011), Q24R has, to our knowledge never been expressed in $[PSI^+]^{Weak}$, and G58D has been reported to strengthen rather than inhibit $[PSI^+]^{Weak}$ (Derkatch et al. 1999). To gain a more complete picture of potential variant-specific effects of these mutants, we determined the phenotypic effect of G58D or Q24R co-expression at different ratios relative to the wildtype protein in $[PSI^+]^{Weak}$ diploids (Fig. 1a, b), as we have previously observed a strong ratio dependency on dominant-negative prion inhibition in $[PSI^+]^{Strong}$ strains (Disalvo et al. 2011).

Q24R displayed a strong dominant-negative inhibition on the $[PSI^+]^{Weak}$ variant, reversing the prion phenotype at a substoichiometric ratio (Fig. 1a,b) and producing dramatic mitotic instability at equal ratios to the wildtype Sup35 (Table 1). Furthermore, when expressed as an additional copy in a haploid $[PSI^+]^{Weak}$ strain (Fig. 2a, c), Q24R resulted in severe dominant-negative inhibition, giving rise to predominantly $[psi^-]$ colonies (Fig. 2a, Table 2). Thus, Q24R expression results in effective dominant-negative interference for both $[PSI^+]^{Strong}$ and $[PSI^+]^{Weak}$ variants (Disalvo et al. 2011).

Previously, Q24R was shown to be inefficient at converting onto wildtype $[PSI^+]^{Strong}$ aggregates *in vivo* by monitoring the appearance of a translation termination defect caused by Sup35 incorporation into aggregates (Satpute-Krishnan and Serio 2005; Disalvo et al. 2011). Unfortunately, as efficient stop codon readthrough is dependent on the unavailability of soluble Sup35, this same assay cannot be performed in $[PSI^+]^{Weak}$ strains due to their accumulation of higher levels of soluble Sup35 (Zhou et al. 1999; Tanaka et al. 2006). However, as the growth of prion aggregates is dependent on the number of templates available for conversion (Ness et al. 2002; Tanaka et al. 2006; Satpute-Krishnan et al. 2007), which falls far below the total number of Sup35 molecules in the cell, agents which act to block this step would be necessary only at substoichiometric ratios relative to wildtype Sup35 (Masel and Jansen 2000). Therefore, the observation that Q24R also inhibits $[PSI^+]^{Weak}$ at substoichiometric ratios suggests that it might also act by decreasing the conversion efficiency of the associated aggregates. Furthermore, the effect of Q24R co-expression is

more severe in $[PSI^+]^{\text{Weak}}$ than in $[PSI^+]^{\text{Strong}}$ strains. The nearly complete reversion to the $[psi^-]$ state in the former would be predicted if Q24R acted to decrease prion conversion efficiency, as $[PSI^+]^{\text{Weak}}$ has fewer propagons, and thus probably fewer surfaces available for conversion, than $[PSI^+]^{\text{Strong}}$ (Cox et al. 2007; Derdowski et al. 2010). Therefore, we suggest that Q24R acts on $[PSI^+]^{\text{Weak}}$ and $[PSI^+]^{\text{Strong}}$ prion conformers similarly, resulting in dominant-negative inhibition by diminishing the conversion efficiency of prion aggregates.

In contrast, the previously reported compatibility of G58D expression with $[PSI^+]^{\text{Weak}}$ cannot be explained by a ratio effect, as G58D did not alter the $[PSI^+]^{\text{Weak}}$ phenotype at any of the ratios tested (Fig. 1a) (Derkatch et al. 1999). However, at equal or sub-stoichiometric ratios, G58D strains had reduced mitotic instabilities than their wildtype counterparts (Table 1), indicative of a prion strengthening effect. These general effects were also observed in a haploid $[PSI^+]^{\text{Weak}}$ strain co-expressing G58D as an additional copy of Sup35, in which G58D co-expression did not result in a phenotypic change (Fig. 2a, c) but slightly enhanced the mitotic stability of the strain (Table 2).

G58D was previously shown to significantly decrease the thermodynamic stability of aggregates in $[PSI^+]^{\text{Strong}}$ haploids and when assembled *in vitro* into fibers at 37°C (Disalvo et al. 2011; Verges et al. 2011). Thus, it seems feasible that G58D incorporation into $[PSI^+]^{\text{Weak}}$ prion aggregates *in vivo* could also lead to their structural destabilization. Because this parameter limits aggregate production through fragmentation and thereby transmission, a G58D-mediated decrease in the thermodynamic stability of these aggregates could explain the reduced

mitotic instability associated with its expression in a $[PSI^+]^{\text{Weak}}$ strain. However our preliminary attempts to directly assess the thermodynamic stability of $[PSI^+]^{\text{Weak}}$ prion aggregates *in vivo* were inconclusive due to high experimental variability and the presence of high levels of soluble Sup35 in $[PSI^+]^{\text{Weak}}$ strains (data not shown).

To determine if G58D was indeed affecting $[PSI^+]^{\text{Weak}}$ aggregates using a different approach, we examined the effect of co-expressing a Q24R/G58D double mutant in $[PSI^+]^{\text{Weak}}$. We had previously observed that combining G58D and Q24R on the same polypeptide exacerbated their dominant-negative inhibition of the $[PSI^+]^{\text{Strong}}$ variant (Disalvo et al. 2011). However in the $[PSI^+]^{\text{Strong}}$ variant, both G58D and Q24R inhibit the prion state in isolation, while in $[PSI^+]^{\text{Weak}}$ variant these mutants differentially affect the prion phenotype (Figs. 1a and 2a). Thus, we reasoned that if G58D did not alter the properties of the prion complex, or only did so insignificantly, the Q24R mutation on the double mutant would still result in effective prion inhibition. However, if G58D promoted $[PSI^+]^{\text{Weak}}$ mitotic stability by enhancing fragmentation of $[PSI^+]^{\text{Weak}}$ aggregates, it should suppress the dominant-negative inhibition of $[PSI^+]^{\text{Weak}}$ by Q24R. In line with the later prediction, co-expression of the Q24R-G58D double mutant in $[PSI^+]^{\text{Weak}}$ essentially rescued the phenotypic reversal of Q24R expression in a $[PSI^+]^{\text{Weak}}$ strain (Fig. 2a, b). Interestingly however, this Q24R-G58D co-expressing strain initially produced a mix of $[psi^-]$ and $[PSI^+]$ colonies, but on subsequent plating of a $[PSI^+]$ colony, the prion phenotype remained stable (Fig. 2b). We interpret this observation to suggest that during the initial growth and

division of the original transformant, a fraction of the population contained aggregates susceptible to Q24R-G58D-induced loss, while those cells retaining $[PSI^+]^{Weak}$ were stabilized by incorporation of the double mutant (Table 2).

Sup35 mutants alter the prion complex

As we found genetic evidence that both G58D and Q24R alter the $[PSI^+]^{Weak}$ state, albeit with different severities and phenotypic outcomes, we went on to determine whether the size distribution of prion complexes exposed to mutant Sup35 expression differed from that of aggregates isolated from cells expressing only wildtype Sup35. To compare the aggregate size distributions in wildtype and mutant co-expressing strains we performed semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) on cell lysates from diploid and haploid $[PSI^+]^{Weak}$ strains expressing wildtype only or co-expressing mutant Sup35 (Kryndushkin et al. 2003). G58D co-expression resulted in ratio-dependent shifts in the size distribution, resulting in a minor or a significant shift towards smaller aggregate size when expressed at sub-stoichiometric ratios or in excess to wildtype Sup35, respectively (Fig. 3a). In contrast, for diploids expressing Q24R even at sub-stoichiometric ratios, the size distribution was shifted towards smaller aggregates (Fig. 3a), similar to its effect on the size distribution of $[PSI^+]^{Strong}$ strains (Disalvo et al. 2011). In line with the high loss of the prion state in cells expressing equal or greater ratios of Q24R to wildtype Sup35, aggregates were completely lost, and only monomeric Sup35 was detected (Fig. 3a).

In contrast, co-expression of both the wildtype and G58D allele in haploid $[PSI^+]^{Weak}$ strains leads to a shift in the size distribution of prion complexes toward larger complexes (Fig. 3b). These results likely reflect both the higher expression of Sup35 in these strains, as we have previously demonstrated (Derdowski et al. 2010), and the limited ability of Hsp104 to fragment $[PSI^+]^{Weak}$ complexes. Furthermore, there appears to be an increase in aggregated Sup35 in the G58D co-expressing strain (Fig. 3b), suggesting that this strain may be propagating more prion complexes than its wildtype counterpart. Consistent with the $[psi^-]$ phenotype of haploid $[PSI^+]^{Weak}$ Q24R co-expressing strains, all the detectable Sup35 is in the monomeric form (Fig. 3b).

Interestingly, co-expression of the double mutant in a haploid strain resulted in a wider size distribution, including the appearance of smaller aggregates (Fig. 2b). Thus, the presence of G58D suppresses the effects of Q24R expression on prion aggregate dynamics. This observation could be explained mechanistically via a number of steps. $[PSI^+]^{Weak}$ strains are characterized by enhanced conversion and reduced fragmentation, which creates both a limitation in the abundance of aggregates and also an increase in their size and decrease in their transmissibility (Tanaka et al. 2006; Derdowski et al. 2010). If Q24R expression decreases conversion efficiency, propagon abundance should further decrease, perhaps falling below the threshold required for maintenance of the $[PSI^+]^{Weak}$ state. If G58D expression enhances fragmentation, it would increase the number of propagons, perhaps elevating their abundance above the threshold and suppressing the effects of Q24R expression.

Mutants differentially alter propagon accumulation

To determine directly if expressing an extra copy of wildtype, G58D, or Q24R/G58D in haploid $[PSI^+]^{Weak}$ strains affected their number of heritable prion units (propagons), we genetically determined these values (Fig. 3c) using an *in vivo* dilution assay (Cox et al. 2003). For the haploid $[PSI^+]^{Weak}$ parent strain, the median number of propagons per cell was approximately 60 (Fig. 3c), and the expression of an additional wildtype copy did not significantly alter propagon abundance (median ~ 40 propagons/cell, Fig. 3c). As we predicted, G58D co-expressing strains displayed a significant increase in the number of propagons compared to its wildtype counterpart, with a median number of 100 propagons per cell (Fig 3c). This increase in propagons could explain the slightly elevated mitotic stability of this strain (Table 2) and is indicative of a stronger $[PSI^+]^{Weak}$ phenotype (Fig 2). Interestingly, the range of propagons per cell for the Q24R-G58D double mutant co-expressing strain overlapped with both the wildtype and G58D expressing strain, suggesting that the enhanced propagon production by G58D correlated with its ability to suppress the dominant-negative effects of Q24R.

G58D inefficiently supports $[PSI^+]^{Weak}$ when expressed as the only copy of Sup35

The ability of G58D to increase the mitotic stability of $[PSI^+]^{Weak}$ propagated by wildtype Sup35, suggests that G58D is not only compatible with this conformer but that it at least partially corrects its defects in prion propagation (Derkatch et al. 1999). However, it is unclear if this corrective effect solely results from the

contributions of G58D or if its interactions with wildtype Sup35 are required. As G58D had been previously shown to support propagation of the $[PSI^+]^{Sc37}$ weak conformation as the sole copy of Sup35 (Verges et al. 2011), we isolated meiotic progeny from the 1WT:1G58D diploid $[PSI^+]^{Weak}$ strain and compared the phenotype and mitotic stability of a G58D haploid with that of its wildtype counterpart. We found that although G58D could support prion propagation when present as the only copy, it produced a weaker phenotype (Fig. 4) and lower mitotic stability than its wildtype counterpart (Table 2). Thus, the variant-specific enhancement of $[PSI^+]^{Weak}$ propagation is a function of mixed prion aggregates.

Discussion

Our studies of Sup35 mutant expression in a $[PSI^+]^{Weak}$ strain demonstrate that prion sequence variants display differential phenotypic effects on distinct prion conformations. Thus, while G58D functions as a dominant-negative inhibitor of $[PSI^+]^{Strong}$ by decreasing the thermodynamic stability of mixed prion complexes, it remains compatible with $[PSI^+]^{Weak}$ prion propagation. In contrast, Q24R is a potent dominant-negative inhibitor of both prion variants. Surprisingly, the double mutant enhances the effects of both single mutants in a $[PSI^+]^{Strong}$ background, while it suppresses inhibition of Q24R in the $[PSI^+]^{Weak}$ background. Although the mechanisms by which these mutants affect $[PSI^+]^{Weak}$ aggregates remains unresolved, it is possible to conceptually explain their phenotypic effects in the $[PSI^+]^{Weak}$ background by extending our mechanistic understanding of their alteration of $[PSI^+]^{Strong}$ aggregates to $[PSI^+]^{Weak}$ aggregates.

First, Q24R effectively inhibits both the weak and strong conformation of $[PSI^+]$. Based on our previous observations demonstrating that Q24R is inefficiently converted by wildtype prion complexes in a $[PSI^+]^{\text{Strong}}$ variant and results in a reduction in propagon accumulation, we hypothesize that the inhibition of $[PSI^+]^{\text{Weak}}$ by Q24R results from Q24R incorporation into $[PSI^+]^{\text{Weak}}$ prion aggregates, which reduces their conversion efficiency. In line with this hypothesis, prion aggregates shift toward smaller size distributions when Q24R is expressed in a $[PSI^+]^{\text{Weak}}$ strain, which is predicted by our hypothesis. Moreover, Q24R is a more effective inhibitor of the $[PSI^+]^{\text{Weak}}$ than the $[PSI^+]^{\text{Strong}}$ variant, abolishing the prion state even when expressed as an additional copy in a $[PSI^+]^{\text{Weak}}$ haploid strain. If Q24R interferes with prion propagation by binding to aggregate templates and decreasing their conversion efficiencies, the fewer templates there are in a given strain, the more effective the inhibition by Q24R will be. Thus, the greater inhibition by Q24R in $[PSI^+]^{\text{Weak}}$ may be due to the accumulation of fewer propagons in $[PSI^+]^{\text{Weak}}$ versus $[PSI^+]^{\text{Strong}}$ prion variants (Cox et al. 2007; Derdowski et al. 2010).

In line with previous observations, G58D expression resulted in opposing phenotypic effects on the $[PSI^+]^{\text{Weak}}$ and $[PSI^+]^{\text{Strong}}$ variants, leading to slightly elevated prion stability versus inducing prion loss, respectively (Disalvo et al. 2011). For the $[PSI^+]^{\text{Strong}}$ variant, G58D incorporation into wildtype prion complexes results in their thermodynamic destabilization, leading to effective prion resolution mediated by Hsp104 under specific cellular conditions. However, G58D is compatible with the $[PSI^+]^{\text{Weak}}$ variant background, displaying no

inhibition of this variant under any of the conditions examined and instead leading to a slight enhancement of its mitotic stability in some strains. Although we did not observe a direct alteration of G58D on the thermodynamic stability of $[PSI^+]^{\text{Weak}}$ aggregates, we cannot rule out this possibility. For example, we monitor aggregate stability as a function of temperature in the presence of SDS. While these conditions are clearly capable of detecting differences between $[PSI^+]^{\text{Weak}}$ and $[PSI^+]^{\text{Strong}}$, they may not detect more subtle changes that could impact the outcome of chaperone engagement with these complexes *in vivo*. Indeed, the altered aggregate size distributions and increased abundance of propagons in G58D co-expressing cells, as well as the unique behavior of the double mutant, suggests that G58D exerts some influence on $[PSI^+]^{\text{Weak}}$ aggregate dynamics. If this is the case, it would explain the elevated mitotic stability of strains expressing G58D, as G58D incorporation could skew the thermodynamic stability of the weak conformer closer to that of the strong conformer. This effect could thereby give $[PSI^+]^{\text{Weak}}$ aggregates more strong-like characteristics without pushing them below the threshold of structural integrity required to evade resolution.

An alternative possibility is that polymorphic sequences could affect the interaction of specific prion conformers with cellular factors, thereby mediating other events in prion propagation *in vivo*. Indeed, in a yeast two-hybrid study, the prion domain of wildtype Sup35, but not that of the G58D mutant, interacts with a cytoskeleton assembly factor Sla1 (Bailleul et al. 1999). Furthermore, the mitotic instability of G58D-only expressing cells in a $[PSI^+]^{\text{Sc4}}$ conformation was rescued

by the overexpression of the Art5 and Lsb3 proteins, which are implicated in endocytosis and actin dynamics, respectively (Verges et al. 2011). However, although these observations have led to the suggestion that G58D expression limits the transmission of prion aggregates to daughter cells in strains replicating the $[PSI^+]^{Sc4}$ strong conformation (Verges et al. 2011) the mother-specific “retention” of propagons reported (Verges et al. 2011) may also be explained by the enhanced frangibility model. In this latter scenario, the limited number of transmitted aggregates is resolved by Hsp104 specifically in daughter cells, an idea for which we now have experimental support (see Appendix).

While it is formally possible that the same Sup35 mutant can evoke prion loss through completely distinct pathways *in vivo*, perhaps a more simplistic explanation is that sequence variants differentially affect the prion phenotype by altering the kinetics of distinct steps of aggregate self-replication. These alterations could result in distinct phenotypic outcomes dependent on the original properties of the underlying prion variant, which would determine the type and extent of the alteration that they can individually withstand. Thus, while a reduction of thermodynamic stability for $[PSI^+]^{Strong}$ may allow for effective aggregate resolution, a similar effect on the thermodynamically more stable weak conformation might enhance prion amplification and produce a stronger phenotype.

The key to resolving this open question is a comparison of the thermodynamic stabilities among naturally occurring prion variants (i.e $[PSI^+]^{Weak}$ and $[PSI^+]^{Strong}$) and their synthetic counterparts ($[PSI^+]^{Sc37}$ and $[PSI^+]^{Sc4}$, respectively). If the

stability of $[PSI^+]^{Sc4}$ lies between that of $[PSI^+]^{Weak}$ and $[PSI^+]^{Strong}$, the *in vivo* effects of G58D expression, which appear to be intermediate between $[PSI^+]^{Weak}$ and $[PSI^+]^{Strong}$, can be rationalized. Specifically, G58D does not act as a dominant negative for $[PSI^+]^{Weak}$ and $[PSI^+]^{Sc4}$, but it does for $[PSI^+]^{Strong}$. Moreover, G58D can partially support $[PSI^+]^{Weak}$ but not $[PSI^+]^{Sc4}$ or $[PSI^+]^{Strong}$ as the only copy of Sup35. Interestingly, the thermodynamic stability of G58D^{Sc37} fibers lies in-between that of the WT^{Sc37} and WT^{Sc4} fibers (Verges et al. 2011), and while G58D^{Sc37} $[PSI^+]$ propagates stably *in vivo*, G58D^{Weak} has reduced mitotic stability in comparison with wildtype Sup35, which could suggest that $[PSI^+]^{Sc37}$ is more thermodynamically stable than $[PSI^+]^{Weak}$. Based on this hypothesis, the final thermodynamic stability of a prion conformation may have to surpass a specific threshold to result in prion destabilization. In contrast, a mutant such as Q24R, which has been suggested to inhibit prion growth, would be predicted to display some degree of prion inhibition on most, if not all, prion variants. In this regard, the comparison between wildtype and mixed prion aggregates could also be informative.

Consistent with the idea that G58D is having a similar effect on $[PSI^+]^{Weak}$ and $[PSI^+]^{Strong}$ variants *in vivo*, the expression of a Q24R-G58D double mutant suppresses the inhibition of $[PSI^+]^{Weak}$ but not of $[PSI^+]^{Strong}$ by Q24R, which is thought to arise due to a defect in propagon production. Thus, this suppression can be conceptually explained by our proposed effects of G58D and Q24R on the properties of the prion complex. For instance, if the conversion efficiency of the weak conformer is inhibited by Q24R, this growth defect may be rescued by

increasing propagon amplification through inducing thermodynamic destabilization via G58D incorporation.

Overall, our results suggest that the phenotypic outcome of prion mutant co-expression is influenced by the properties of the underlying prion conformer. While our phenotypic observations of mutant co-expression in $[PSI^+]^{\text{Weak}}$ strains can be explained through a mechanism whereby Q24R and G58D inhibit the conversion efficiency or enhance prion frangibility of prion complexes, respectively, additional biochemical experiments have the potential to further validate this model.

Table 1: Spontaneous frequencies of $[PSI^+]$ loss during mitotic division in diploid strains

Strain	$[PSI^+]$ Loss (% $[psi^-]$)	Colonies Scored
SLL3071 ($[PSI^+]^{Strong}$)	0.035	2831
SY945 ($[PSI^+]^{Weak}$)	0.29	4077
SY1773 (3WT)	0.26	2681
SY1774 (2WT:1G58D)	0	1807
SY1775 (2WT:1Q24R)	0.58	1865
SY1776 (2WT)	0.15	3982
SY1777 (1WT:1G58D)	0.07	2981
SY1778 (1WT:1Q24R)	88.13	1769
SY1779 (3WT)	0.35	1989
SY1780 (1WT:2G58D)	0.36	4175
SY1781 (1WT:2Q24R)	98.24	2439

Table 2: Spontaneous frequencies of $[PSI^+]$ loss during mitotic division in haploid strains

Strain	$[PSI^+]$ Loss (% $[psi^-]$)	Colonies Scored
SLL2606 ($[PSI^+]$ Strong)	0	3850
SLL2600 ($[PSI^+]$ Weak)	0.614	4232
SY1809 (WT+WT)	0.1	4628
SY1810 (WT+G58D)	0.057	3480
SY1811 (WT+Q24R)	94.5	1481
SY2000 (WT+Q24RG58D)	10-0.5*	1310
SY2026 (G58D only)	12.65	324

Table 3: Plasmids

Name	Description
6686	pRS306-P ₃₅ Sup35
SB467	pRS306-P ₃₅ Sup35(G58D)
SB410	pRS306-P ₃₅ Sup35(Q24R)
SB644	pRS304P ₃₅ Sup35
SB645	pRS304P ₃₅ Sup35(G58D)
SB646	pRS304P ₃₅ Sup35(Q24R)
SB657	pRS306-P _{tetO2} Sup35
SB658	pRS306-P _{tetO2} Sup35(G58D)
SB659	pRS306-P _{tetO2} Sup35(Q24R)
SB733	pRS306-P _{tetO2} Sup35(Q24R, G58D)

Table 4: Yeast Strains

Strain	Genotype	Plasmids integrated	Described in figure	Reference
74-D694	<i>MATa</i> (or <i>MATa</i> or <i>MATa/α</i>) [<i>PSI</i> ⁺] (or [<i>psi</i>]) <i>ade1-14 his3Δ200 trp1-289 ura3-52 leu2-3,112</i>	-	1, 2, 3, 4	Chernoff et al. 1995
SY1773	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112</i>	6686	1, 3a	This study
SY1774	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112</i>	SB467	1, 3a	This study
SY1775	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112</i>	SB410	1, 3a	This study
SY1776	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	6686	1, 3a	This study
SY1777	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB467	1, 3a	This study
SY1778	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB410	1, 3a	This study
SY1779	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52 /ura3-52::URA3::P₃₅SUP35 leu2-3,112/ leu2-3,112</i>	6686	1, 3a	This study
SY1780	<i>MATa/α</i> [<i>PSI</i> ⁺] ^{Weak} <i>ade1-14/ade1-14 his3Δ200/ his3Δ200</i>	SB467	1, 3a	This study

	<i>trp1-289/trp1-289::TRP1::P₃₅SUP35(G58D) ura3-52/ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB645		
SY1781	<i>MATa [PSI⁺]^{Weak} ade1-14/ade1-14 his3Δ200/ his3Δ200 trp1-289/trp1-289::TRP1::P₃₅SUP35(G58D) ura3-52/ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB410 SB646	1, 3a	This study
SY1809	<i>MATa [PSI⁺]^{Weak} ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35 leu2-3,112</i>	SB657	2, 3b,c	This study
SY1810	<i>MATa [PSI⁺]^{Weak} ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3,112</i>	SB658	2, 3b,c	This study
SY1811	<i>MATa [PSI⁺]^{Weak} ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24R) leu2-3,112</i>	SB659	2, 3b	This study
SY2000	<i>MATa [PSI⁺]^{Weak} ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24R-G58D) leu2-3,112</i>	SB733	2, 3b,c,	This study
SY2026	<i>MATa [PSI⁺]^{Weak} ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P₃₅SUP35(G58D) leu2-3,112 SUP35/sup35::KANMX4</i>	SB645	4	This study

Table 5: Primers

SD27 5' ACTTGCTCGGAATAACATCTATATCTGCCCACTAGCAACACAGC
TGAAGCTTCGTACGC 3'

SD28 5'GGTATTATTGTGTTTGCATTTACTTATGTTTGCAAGAAATGCATA
GGCCACTAGTGGATCTG 3'

Methods

Plasmid Construction

All plasmids used in this study are listed in Table 3. See (Disalvo et al. 2011) for plasmid generation.

Strain Construction. All yeast strains are derivatives of 74-D694 and are listed in Table 4. Yeast strains expressing ectopic copies of *SUP35* or PNM mutants from *URA3* (pRS306) or *TRP1* (pRS304)-based plasmids were constructed by transforming yeast strains with plasmids that were linearized with *Bst*BI, or *Bsu*361, respectively, and by selecting for transformants on the appropriate minimal medium. In all cases, expression was confirmed by quantitative immunoblotting for Sup35. Disruptions of *SUP35* (SD27, SD28) were generated by transformation of PCR-generated cassettes using pFA6aKanMX4 as a template with the indicated primers (see Table 5) and selection on complete media supplemented with G418 (300µg/ml).

Protein Analysis. Semi-denaturing agarose gel electrophoresis, SDS-PAGE, quantitative immunoblotting, and SDS-sensitivity were performed as previously described (Pezza et al. 2009).

Propagon Counts. The number of propagons per cell was determined by an *in vivo* dilution, colony-based method, as previously described (Cox et al. 2003)

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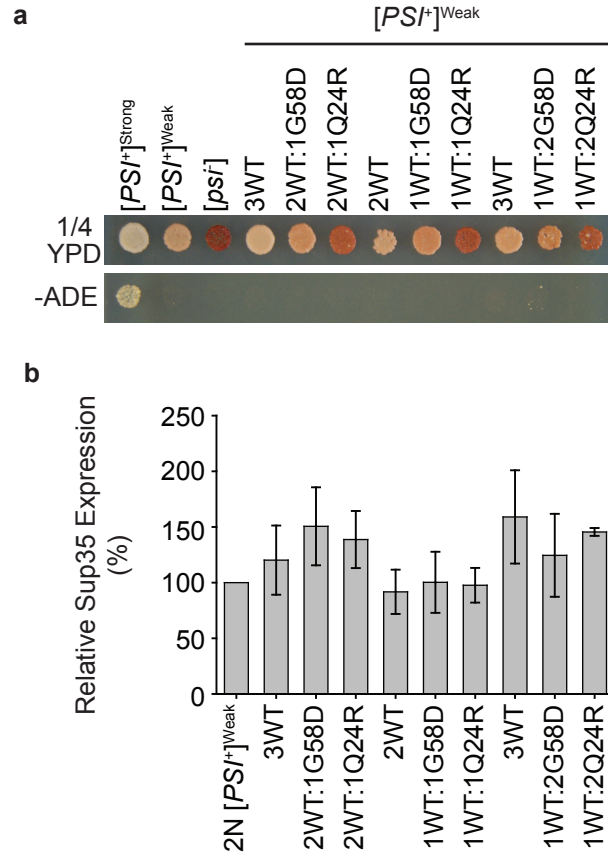


Figure 1. PNM mutants are distinguished by their inhibition of a $[PSI^{+}]^{Weak}$ variant. **a.** Diploid $[PSI^{+}]^{Weak}$ strains expressing wildtype (WT) and PNM mutants (Q24R, G58D) from P_{35} at the indicated ratios were spotted on rich (1/4 YPD) or adenine deficient (-ADE) medium to analyze the $[PSI^{+}]$ phenotype. Wildtype $[PSI^{+}]^{Strong}$, $[PSI^{+}]^{Weak}$, and $[psi^{-}]$ diploids (74-D694) were included as controls. **b.** Lysates isolated from strains described in (a) were analyzed by SDS-PAGE and quantitative immunoblotting for Sup35. A wildtype diploid (2N $[PSI^{+}]^{Weak}$) is included as a control. Expression levels are consistent with Sup35 copy number. Error bars represent standard deviation ($n \geq 3$).

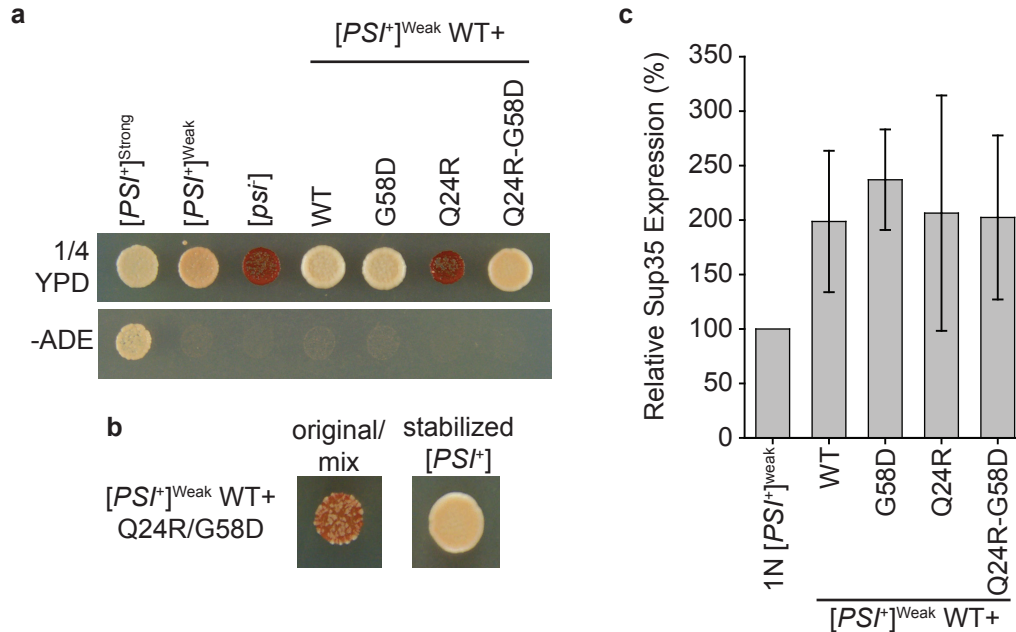


Figure 2. PNM mutants differentially affect the [PSI⁺]Weak phenotype in haploid strains. **a.** Haploid [PSI⁺]Weak strains expressing an additional wildtype (WT) or PNM mutant (Q24R, G58D) from P_{tet02} were spotted on rich (1/4 YPD) or adenine deficient (-ADE) medium to analyze the [PSI⁺] phenotype. Wildtype [PSI⁺]Strong, [PSI⁺]Weak, and [psi⁻] haploids (74-D694) were included as controls. **b.** Original isolate of Q24R-G58D double mutant and a subsequent stable [PSI⁺] colony from haploid [PSI⁺]Weak strain described in (a) were spotted on rich media to analyze the [PSI⁺] phenotype. **c.** Lysates isolated from strains described in (a) were analyzed by SDS-PAGE and quantitative immunoblotting for Sup35. A wildtype haploid (1N [PSI⁺]Weak) is included as a control. Expression levels are consistent with Sup35 copy number. Error bars represent standard deviation (n ≥ 3).

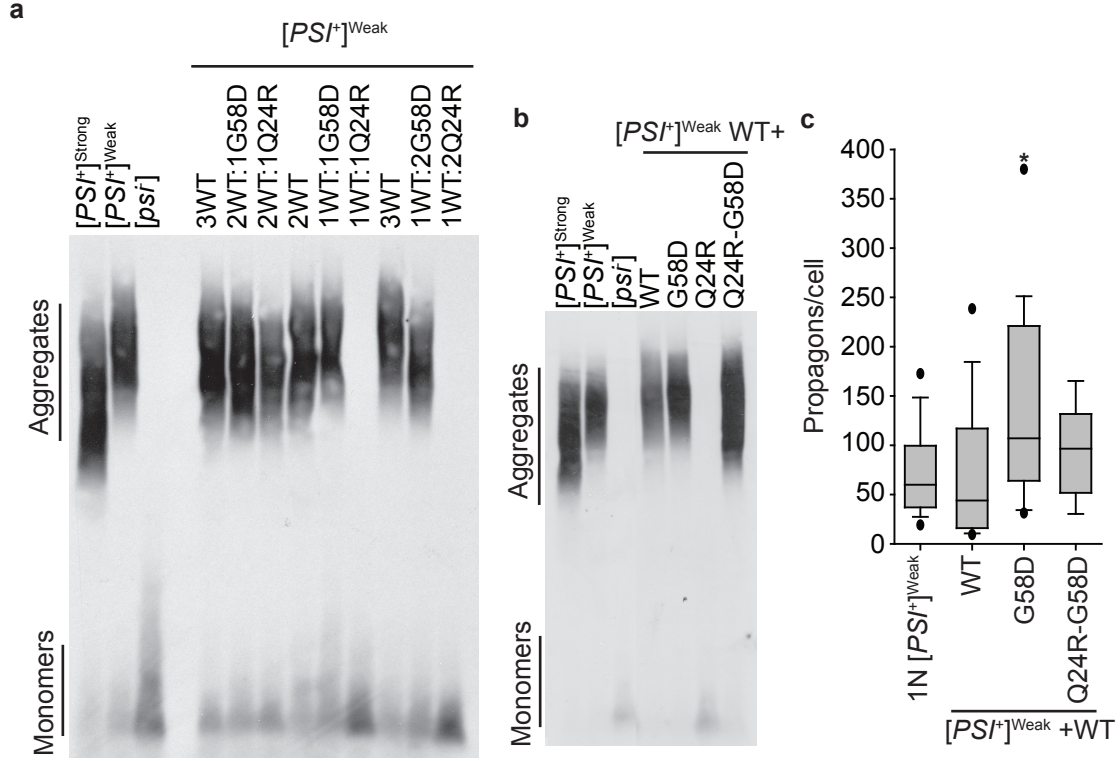


Figure 3. Prion complexes display differential characteristics in mutant expressing strains. a. Lysates isolated from Diploid $[PSI^{+}]^{Weak}$ strains expressing wildtype (WT) and PNM mutants (Q24R, G58D) from P_{35} at the indicated ratios were analyzed by SDD-AGE and immunoblotting for Sup35 to examine their aggregate size distributions. b. Aggregate size distributions for haploid $[PSI^{+}]^{Weak}$ strains co-expressing wildtype (WT) or mutant (Q24R, G58D, Q24R-G58D) from P_{teto2} were examined as described in (a). c. The number of propagons present in individual cells was determined for the indicated strains, as described in (b). Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles; error bars indicate 10th and 90th percentiles, and dots represent outliers ($n \geq 10$; * $p = 0.0028$ and 0.0004 in comparison with WT and 1N $[PSI^{+}]^{Weak}$ parent respectively).

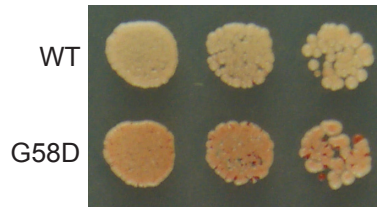


Figure 4. G58D-only $[PSI^+]$ ^{Weak} strains produce a less stable phenotype than wild-type $[PSI^+]$ ^{Weak}. Haploid $[PSI^+]$ ^{Weak} strains expressing wildtype (WT) or G58D (G58D) from P_{35} were spotted on rich (1/4 YPD) medium to analyze the $[PSI^+]$ phenotype.

Chapter 4: Discussion

**The Mechanism of Dominant-Negative Prion Inhibition Reveals the Crucial
Interplay between Protein Misfolding and its Cellular Environment**

Investigating the Mechanism of Prion Inhibition

Prion Propagation and Metastability

According to the prion hypothesis, an alternative conformation of a normal, host-encoded protein, known as a prion, can function as a genetic determinant of transmissible phenotypic states. *In vitro* studies of prion conformational selection and amplification demonstrate that the assembly of prion conformers into amyloid aggregates directs the continued misfolding of the protein by providing a template for this conversion (Kocisko et al. 1994; Glover et al. 1997; King et al. 1997; Taylor et al. 1999; Sondheimer and Lindquist 2000; Saborio et al. 2001; Dos Reis et al. 2002). However, studies on the yeast prion Sup35 indicate that the process of conformational self-replication is more complex *in vivo* (Fig. 1, top), and a similar pathway has been proposed for prion propagation in mammals (Masel et al. 1999). Based on this work, aggregates or their component prion-state protein must act in three roles to sustain the prion-associated phenotype in a population. As is the case *in vitro*, these aggregates template the conversion of newly made Sup35 to the prion conformer (Patino et al. 1996; Satpute-Krishnan and Serio 2005), but these aggregates must also be continually fragmented *in vivo* to create new conversion surfaces and to be transmitted to daughter cells (Fig. 1, top) (Satpute-Krishnan et al. 2007; Derdowski et al. 2010).

While highly efficient, this pathway of prion propagation allows for phenotypic metastability, with the potential for new traits to arise, persist, adapt, or disappear within an organism's lifetime. This metastability results from even slight

modifications in the efficiencies of any of the events in the prion cycle. For example, prion sequence polymorphisms, which confer protection against prion infection, can dominantly inhibit propagation of the prion state *in vivo* (Kaneko et al. 1997; Zulianello et al. 2000; Perrier et al. 2002; Crozet et al. 2004; Atarashi et al. 2006; Furuya et al. 2006; Ott et al. 2008; Toupet et al. 2008; Hizume et al. 2009), and changes to the amino acid sequence of the yeast Sup35/[PSI⁺] prion can impair the maintenance and propagation of the [PSI⁺] state (Doel et al. 1994; DePace et al. 1998; Kochneva-Pervukhova et al. 1998). Because these sequence mutants act dominantly, they likely compromise crucial events in prion propagation by the wildtype protein. Thus, I reasoned that elucidating their mechanisms of action should illuminate the dynamic processes underlying prion metastability *in vivo* and could be instructive for developing therapeutic interventions for prion diseases (Crozet et al. 2004; Furuya et al. 2006; Toupet et al. 2008).

Yeast [PSI⁺]-No-More Mutants

The [PSI⁺] system is ideally suited for studying prion propagation *in vivo*, making it a valuable model in which to probe the effects of dominant-negative prion inhibition. Sup35 prion-domain mutants that induce [PSI⁺]-to-[psi⁻] reversion when transiently co-expressed with wildtype Sup35 are termed PNM for [PSI⁺]-No-More (Doel et al. 1994). Although a few yeast PNM mutants have been characterized by other groups, their precise mechanisms of dominant-negative inhibition have remained elusive (Kochneva-Pervukhova et al. 1998; Derkatch et al. 1999).

Based on their dominant effects on wildtype Sup35, I hypothesized that PNM mutants abrogated prion propagation by incorporating into and altering Sup35 aggregates in a way that compromises the efficiency of their *in vivo* biogenesis cycle (Fig 1, top). Therefore, I investigated the effect of co-expressing wildtype and the dominant-negative Sup35 mutants G58D or Q24R on prion aggregates *in vivo* to elucidate the mechanisms underlying prion dominant inhibition. I found that these mutants were effective at distinct inhibitory ratios and used a series of *in vivo* analyses to link these differences to defects in discrete events in the prion propagation pathway. Although both mutants could incorporate into wildtype prion complexes, they differentially altered the properties of these aggregates. Due to these alterations, mixed complexes became sensitive to resolution by the molecular chaperone Hsp104, resulting in effective cellular intervention and prion destabilization.

Dominant-negative inhibition by G58D

G58D reduces aggregate thermodynamic stability, allowing for effective chaperone-mediated prion clearance

Based on my studies, G58D only significantly interferes with prion propagation in the $[PSI^{+}]^{\text{Strong}}$ variant at higher ratios relative to wildtype protein, suggesting that its incorporation throughout the aggregate is required for its dominant effects (Masel and Jansen 2000). Consistent with this idea, G58D was conversion-competent, an activity mediated solely by aggregate ends, although a minor defect in this reaction was revealed by careful analysis of fluorescence intensity produced from a stop codon readthrough-reporter, which depends upon the

translation termination defect caused by Sup35 incorporation into aggregates (DePace et al. 1998; Kochneva-Pervukhova et al. 1998; Derkatch et al. 1999; Osherovich et al. 2004; Satpute-Krishnan and Serio 2005). In contrast, incorporation of G58D into wildtype prion complexes lowered their thermodynamic stability as measured by their sensitivity to disruption in SDS at increasing temperatures, a property of the intermolecular interactions among the monomers throughout the complex (chapter 2). Thus, G58D expression likely increases fragmentation efficiency.

An increase in fragmentation efficiency might also explain the compatibility of G58D expression with $[PSI^+]^{Weak}$ (chapter 3) (Derkatch et al. 1999), which accumulates fewer aggregates due to its enhanced thermodynamic stability (Tanaka et al. 2006). Although a reduction of thermodynamic stability of $[PSI^+]^{Weak}$ by G58D was not directly demonstrated by my studies, it is possible that a subtle shift in this parameter, perhaps outside of the sensitivity of our analyses, is sufficient to produce the increase in propagons and the enhanced mitotic stability (Derkatch et al. 1999) observed for $[PSI^+]^{Weak}$ haploids co-expressing G58D. Consistent with this prediction, *in vitro* studies from the Weissman group demonstrate that G58D fibers of a particular conformation (Sc37), which supports a $[PSI^+]^{Weak}$ phenotype *in vivo*, display a mildly reduced thermodynamic stability (Verges et al. 2011).

Perhaps providing the strongest support for a mechanism whereby G58D promotes fragmentation, its dominant-negative effects were not a function solely of its own expression. Rather, they were modulated by the cellular environment,

with propagon accumulation increasing in $[PSI^+]^{\text{Strong}}$ haploid strains expressing G58D but decreasing in $[PSI^+]^{\text{Strong}}$ diploid strains. These differences could be explained by the relative doses of Sup35 and Hsp104 under the two conditions (2:1 vs. 1:1, respectively). Indeed, the dominant-negative effect of G58D was completely reversed by lowering Hsp104 levels and enhanced by even mild overexpression of Hsp104. Moreover, we found differences in the Sup35:Hsp104 ratio in yeast strains of different genetic backgrounds (chapter 2), which provided a molecular explanation for known variations in G58D efficacy (Doel et al. 1994; Derkatch et al. 1999).

The dependency of prion inhibition on chaperone level suggested that expression of dominant-negative mutants could promote the disassembly of aggregates *in vivo*, and we directly observed this process by monitoring the fate of existing Sup35 in yeast strains after treatment with cycloheximide. In these experiments, Sup35 transitioned from an SDS-resistant to an SDS-sensitive form in yeast strains expressing both wildtype and G58D but not in those expressing wildtype protein alone. Mechanistically, we predict that G58D induces this outcome by enhancing the efficiency of the Hsp104 fragmentation reaction (Fig. 1, middle). Thus, expression of G58D skews the competition between aggregate assembly and disassembly pathways toward the latter, allowing the effective clearance of an established prion conformer *in vivo*.

Other proposed mechanisms for curing by G58D

Although our results suggest a model whereby G58D co-expression in $[PSI^+]^{\text{Strong}}$ strains produces less thermodynamically stable aggregates that are sensitive to

cellular resolution, work from the Weismann group suggested an alternative curing mechanism for G58D when replicating another $[PSI^+]^{\text{Strong}}$ conformer (Sc4) (Verges et al. 2011). These authors produced homotypic WT or G58D fibers *in vitro* using conditions which produce a strong phenotype ($[PSI^+]^{\text{Sc4}}$) when introduced into yeast cells and examined the *in vitro* thermodynamic stability and conversion rates of these fibers and their effect on the prion phenotype *in vivo*.

Interestingly, G58D^{Sc4} fibers did not display altered thermodynamic stability or conversion efficiency compared to their wildtype counterparts *in vitro*, despite their impaired prion propagation, as assessed by colony color, when expressed as the only copy of Sup35 *in vivo*. Although overexpression of Hsp104 increased prion loss in G58D $[PSI^+]^{\text{Sc4}}$ haploids, the Weissman group did not detect an increase in Sup35 fragmentation, using a mutated form of Hsp104 that associates with an inactive form of the bacterial protease ClpP. In this system, substrates that have been translocated through the central pore of Hsp104 are trapped in ClpP (Tessarz et al. 2008).

While these experiments have clear caveats (see below), the Weissman group proposed an alternative mechanism for inhibition of $[PSI^+]^{\text{Sc4}}$ propagation by G58D: a defect in partitioning of prion aggregates to daughter cells during division. Consistent with this idea, G58D $[PSI^+]^{\text{Sc4}}$ strains occasionally produced daughter cells without propagons, and overexpression of LSB3 and ART5, two factors implicated in actin dynamics and/or endocytosis (Madania et al. 1999; Lin et al. 2008; Nikko and Pelham 2009; Robertson et al. 2009; Tonikian et al. 2009) minimized prion loss. Interestingly, a previous Sup35 yeast-two hybrid screen

found that the prion domain of wildtype Sup35, but not that of G58D, interacted with Sla1, which also assists in actin microfilament formation (Ayscough et al. 1999; Bailleul et al. 1999). Together, these observations were taken as evidence of an active, actin-mediated process for aggregate partitioning, and somehow G58D specifically compromised this pathway for Sup35 aggregates in the $[PSI^+]^{Sc4}$ conformation.

Reconciling conflicting observations of G58D prion inhibition

While it is possible that G58D expression inhibits propagation of $[PSI^+]^{Strong}$ and $[PSI^+]^{Sc4}$ through completely different pathways, the discrepancy between the two models can potentially be explained in several ways. First, while my studies of G58D investigated dominant-negative inhibition, Verges et al. primarily examined the inefficient propagation of $[PSI^+]^{Sc4}$ strains expressing G58D as the sole form of Sup35. Therefore, it is possible that aggregate association with cellular co-factors is different when G58D is expressed alone or in combination with wildtype Sup35. For instance, if G58D did not interact with a co-factor required for transmission, co-expression of wildtype Sup35 would be predicted to rescue this inefficient interaction. In contrast, if G58D interacted with a new cellular co-factor as a gain of function that restricted its transmission, this interaction should still occur in strains co-expressing wildtype Sup35, but the degree of this interaction would likely be influenced by the ratio of the two proteins. In either of these scenarios and in light of our observations of G58D effects on conversion and fragmentation, G58D could impact multiple steps of aggregate biogenesis, but

the predominant defect would be influenced by the relative ratios of mutant and wildtype proteins.

Another possibility is that the relationship between G58D and the underlying prion conformer could influence its mechanism of curing. For instance, G58D displays different effects on distinct prion variants, which could be due to its ability to alter aggregate stability under some conditions but not others. Therefore, prion loss could potentially occur through distinct mechanisms dictated by the susceptibility of prion conformers to particular types of aggregate alteration. That our $[PSI^+]^{\text{Strong}}$ and $[PSI^+]^{\text{Weak}}$ variants may be distinct from $[PSI^+]^{\text{Sc4}}$ and $[PSI^+]^{\text{Sc37}}$ respectively, is suggested from our observations that G58D does not efficiently support $[PSI^+]^{\text{Weak}}$ when present as the only copy (chapter 3), in contrast to its ability to stably propagate $[PSI^+]^{\text{Sc37}}$ as the only copy. To directly compare the effects of G58D expression on these distinct conformations in the same context, one could perform the same series of *in vivo* experiments on $[PSI^+]^{\text{Sc4}}$ and $[PSI^+]^{\text{Sc37}}$ that I have conducted for $[PSI^+]^{\text{Strong}}$ and $[PSI^+]^{\text{Weak}}$ variants to determine if their behavior is truly distinct.

Despite these possible explanations for the discrepancies between our models, several findings by Verges *et al.* may actually be consistent with the mechanism that we report for dominant-negative inhibition: G58D enhances the frangibility of prion complexes to allow for effective Hsp104 mediated intervention. First, the propagon partitioning data, used as the primary support for a transmission defect, can also be interpreted to suggest that G58D prion complexes are more effectively resolubilized. According to the reported observations on some mother-

daughter pairs, propagons are only recovered from the mother cell (Verges et al. 2011), possibly through a defect in inheritance. However, other mother-daughter pairs display an opposing trend, with more propagons recovered from daughters in relation to their mothers, a phenomenon not observed in wildtype strains and clearly inconsistent with a transmission defect. In contrast, both of these phenomena can be successfully explained by enhanced fragmentation of G58D aggregates. For instance, if daughter cells receive propagons that are more efficiently fragmented by molecular chaperones, fragmentation may result in aggregate resolubilization or propagon amplification depending on the relative amount of transmitted aggregate and Hsp104. Consistent with this scenario, inhibition of fragmentation by Gdn treatment specifically in daughter cells reverses prion loss (see appendix). To determine if the increase in propagon numbers in daughter cells is due to more effective amplification by Hsp104, one could compare the propagon distributions from mother-daughter pairs in which Hsp104 function has been inhibited either pre or post-budding.

Additionally, the finding by Verges *et al.* that moderate overexpression of Hsp104 (2-3 fold) strongly destabilizes the G58D $[PSI^+]^{Sc4}$ strain but only modestly destabilized WT $[PSI^+]^{Sc4}$ if at all, is consistent with enhanced resolubilization of G58D prion complexes. However, the authors also found that overexpression of an Hsp104 mutant, which lacks the N-terminus (Δ NTD) and is functional in prion propagation and thermotolerance but does not cure $[PSI^+]$ when overexpressed (Hung and Masison 2006), did not increase prion loss in the G58D strain. Because Hsp104 Δ NTD overexpression did not lead to loss of G58D-dependent

[*PSI*⁺], the authors conclude that the mechanism of prion curing by overexpression of wildtype Hsp104 cannot result from aggregate resolubilization. However, two important considerations must influence the interpretation of these results. First, the activity of Hsp104ΔNTD is not well characterized and may differ from that of wildtype Hsp104. Second, prion curing of mutant expressing strains is detected at moderate levels of Hsp140 overexpression (around 2.5 fold; chapter 2), while wildtype prion loss only occurs when Hsp104 is highly overexpressed (over 4-5 fold) (C. Klaips unpublished observations).

In line with the possibility that Hsp104ΔNTD may have differential fragmentation activity, expressing it as the only copy of Hsp104 produces a more severe [*PSI*⁺] phenotype (Hung and Masison 2006; Verges et al. 2011). In order to determine the relative fragmentation efficiency of Hsp104ΔNTD one could compare prion aggregate size distributions in wildtype Hsp104 and Hsp104ΔNTD expressing strains. If Hsp104ΔNTD expressing strains contain prion complexes with a size distribution that is shifted toward larger complexes, this observation would suggest that Hsp104ΔNTD is less efficient at prion fragmentation, despite its ability to still propagate the prion state. Furthermore, it would be informative to quantify prion loss in WT [*PSI*⁺]^{Sc4} and G58D [*PSI*⁺]^{Sc4} strains overexpressing wildtype Hsp104 versus Hsp104ΔNTD at different levels, as the effects of these treatments have been only qualitatively described. This analysis would allow us to better compare prion loss between the two strains with the relative activities of the Hsp104 molecules expressed. Additionally, as Hsp104ΔNTD functions in thermotolerance and we observe an increase in prion loss in mutant co-

expressing strains after heat-shock (see appendix), it would be interesting to determine whether [*PSI*⁺] is similarly destabilized by heat-shock in comparable Hsp104 Δ NTD strains or if this route of prion curing is also compromised by deletion of the Hsp104 N-terminus.

Regarding the mechanism of curing by overexpression of Hsp104, it should be noted that wildtype [*PSI*⁺] strains are only cured by very high levels of Hsp104 overexpression, while moderate Hsp104 overexpression does not induce prion loss despite reversing the prion phenotype (less readthrough) (Chernoff et al. 1995). This observation suggests that moderate Hsp104 overexpression may increase the level of soluble Sup35 presumably through enhanced prion fragmentation, but this fragmentation elevation remains below the threshold required for complete aggregate resolution. Nonetheless, the same moderate levels of Hsp104 overexpression are sufficient to enhance prion loss in G58D co-expressing strains (chapter 2). Our interpretation for this difference is that enhanced disassembly of wildtype prion aggregates via elevated chaperone levels may be compensated for by the high efficiency of the aggregate assembly pathway, but if the assembly pathway is already compromised (by Q24R) (see dominant negative inhibition by Q24R), or the disassembly pathway is already favored (by G58D), then further disassembly enhancement could lead to complete prion complex dissolution. In order to determine if the conversion efficiency of wildtype Sup35 prion complexes prevents their resolution by moderate levels of Hsp104, one could decrease the expression of Sup35 by putting it under a titratable promoter (to decrease its corresponding conversion

rate) and test its susceptibility to prion loss during different levels of Hsp104 overexpression.

Dominant-negative inhibition by Q24R

In contrast to the G58D mutant, our studies of Q24R co-expression suggest that this mutant leads to prion loss by incorporating into wildtype prion aggregates and inhibiting prion conversion. First, Q24R interferes with prion propagation at sub-stoichiometric ratios for both a $[PSI^{+}]^{\text{Strong}}$ and $[PSI^{+}]^{\text{Weak}}$ variant, consistent with interference with an activity mediated through a limiting number of sites on aggregates, such as the two growth-compatible surfaces. Second, Q24R displayed only half the conversion efficiency of WT Sup35. Consistent with an aggregate growth defect, Q24R expression decreased propagon accumulation and resulted in smaller prion aggregates in a $[PSI^{+}]^{\text{Strong}}$ strain, and lower levels of Q24R expression were sufficient to inhibit propagation of the $[PSI^{+}]^{\text{Weak}}$ variant, which accumulates significantly fewer aggregates than $[PSI^{+}]^{\text{Strong}}$ (chapter 3) (Tanaka et al. 2006).

Loss of prion aggregates in cells co-expressing Q24R could ultimately occur in several ways. First, reduction in aggregate abundance could lead to aggregate dilution during cell division, eventually resulting in a lack of prion transmission to some cells (also see: Differential cellular conditions modulate the prion state within cellular populations). Thus, the rate of cell growth could outpace the rate of aggregate accumulation and transmission (Fig. 1, middle). Additionally, like G58D, Q24R could induce prion loss by enhancing the aggregate disassembly pathway. In support of this idea, we found that aggregates in Q24R co-

expressing strains could also be resolubilized: while monitoring the fate of existing Sup35 in Q24R co-expressing strains after treatment with cycloheximide, Sup35 transitioned from an SDS-resistant to an SDS-sensitive form. Mechanistically, we predict that Q24R induces this outcome by increasing the ratio of enzyme (Hsp104) to substrate (aggregates). Thus, in addition to reducing aggregate accumulation and thereby inheritance, expression of Q24R can also enhance the disassembly of prion aggregates to allow for effective prion clearance. Consistent with this model, the dominant-negative effects of Q24R expression were partially reversed by lowering Hsp104 levels and were enhanced by overexpression of Hsp104.

One unresolved question is how Q24R inhibits conversion of wildtype Sup35 mechanistically. Conceptually, Q24R must bind to aggregate ends and subsequently block or slow the incorporation of the wildtype protein. This effect could be accomplished simply by a lower affinity between the proteins (i.e., wildtype with Q24R and Q24R with Q24R) such that, once Q24R becomes incorporated, wildtype Sup35 is then less efficiently recruited to the fiber ends. It is also possible that Q24R binds to fiber ends and is inefficiently converted (i.e. conformationally remodeled) to the prion state, which would slow the overall reaction and thereby transiently block this surface to further conversion events. In either scenario, the incorporation of Q24R into prion aggregates must be efficient enough to allow its distribution along the length of the fiber, as fragmentation will continually generate new ends. Consistent with this prediction,

Q24R, like G58D, inhibits wildtype prion propagation more efficiently as its expression level is raised.

To resolve these remaining mechanistic questions, a series of *in vitro* and *in vivo* experiments could be performed. To determine if Q24R inhibits wildtype conversion, one can examine whether Q24R can be assembled into fibers *in vitro*, and, if so, determine if these complexes can efficiently seed conversion of wildtype Sup35. Alternatively, one can measure the conversion of Q24R onto wildtype prion fibers *in vitro*. To determine if Q24R binds to aggregate ends but is inefficiently converted to the prion state *in vivo*, one could precipitate aggregates from Q24R co-expressing strains via centrifugation and determine their SDS solubility to assess whether all of the aggregated protein has been converted into an SDS-insoluble conformation (Chen et al. 2010).

Q24R-G58D double-mutant inhibition

Interestingly, the double mutant Q24R-G58D also differentially affects $[PSI^+]$ prion variants. Its expression in a $[PSI^+]^{\text{Strong}}$ strain significantly inhibits the prion state, doing so more effectively than either single mutant alone. However, in a $[PSI^+]^{\text{Weak}}$ strain, the presence of G58D actually appears to suppress the effect of Q24R: while Q24R effectively inhibits prion propagation in $[PSI^+]^{\text{Weak}}$, Q24R-G58D co-expression results in cells with similar stability and propagon numbers as the $[PSI^+]^{\text{Weak}}$ wildtype state. Therefore, when both mutants individually inhibited the propagation of a prion variant, the double mutant enhanced their phenotypic effects, but if the single mutants had opposing effects on the

propagation of a prion variant, the double mutant effectively cancels out these effects.

These observations could be explained mechanistically by the effects of mutant incorporation into prion aggregates of distinct conformations. Additionally, they might support the hypothesis that there is a minimum threshold of aggregate size and number required for efficient prion maintenance, which is perhaps already implied by the ratio dependency of dominant-negative inhibition. As $[PSI^+]^{\text{Strong}}$ variants have more and smaller aggregates with lower thermodynamic stabilities than $[PSI^+]^{\text{Weak}}$ variants (Kryndushkin et al. 2003; Pezza et al. 2009; Derdowski et al. 2010), slowing their growth and simultaneously enhancing the fragmentation of the former could more effectively lead to their resolution. It may be worthwhile to determine the propagon numbers and aggregate sizes of $[PSI^+]^{\text{Strong}}$ strains co-expressing Q24R-G58D as I would predict them to be fewer and smaller than aggregates from strains co-expressing either mutant alone. $[PSI^+]^{\text{Weak}}$ variants, on the other hand, have more soluble Sup35 and fewer, larger aggregates, which are more thermodynamically stable (Uptain et al. 2001; Kryndushkin et al. 2003; Pezza et al. 2009; Derdowski et al. 2010). Therefore, the conversion inhibition of Q24R could be compensated for by the generation of more prion aggregates (and conversion templates) by G58D, producing aggregates with sizes and numbers above the required minimum threshold. If this is true, it may be possible to dip these complexes below the threshold by co-expressing the double mutant at higher levels than the wildtype protein in $[PSI^+]^{\text{Weak}}$ strains.

Competition between aggregate assembly and disassembly pathways

A wealth of $[PSI^+]$ studies have demonstrated that efficient fragmentation is essential for prion propagation, with larger and more thermodynamically stable prion variants having lower mitotic stabilities (Derkatch et al. 1996; Derdowski et al. 2010). While my studies of dominant negative-inhibition by G58D and Q24R are the first, to my knowledge, directly demonstrating that alterations to prion aggregates, which enhance prion fragmentation, can also lead to prion loss. Thus, a “safe-zone” of aggregate fragmentation efficiency exists in which the prion state is stable, while aggregates with properties outside of this zone at either extreme are susceptible to resolubilization or inefficient transfer, respectively (Fig. 2). Therefore, the aggregate assembly and disassembly pathways must be finely balanced to allow for efficient prion propagation.

Differential cellular conditions modulate the prion state

Daughters are more susceptible to prion loss

Given that prion loss by G58D either requires or is exacerbated by cell division (McCready et al. 1977; Cox 1993) and that phenotypic heterogeneity, correlating with differential accumulation of aggregates among cells, exists within a population (Byrne et al. 2009; Derdowski et al. 2010), we hypothesized that a specific subset of the population might be more susceptible to prion loss by dominant-negative mutants. In line with the inheritance of fewer propagons by daughter cells, especially from new mothers (Fig. 3a), we found that daughters reverted to the $[psi^-]$ state significantly more frequently (approximately 0.5 fold) than cells from the total population upon co-expression of G58D or Q24R. Interestingly, this increase in prion loss by daughters in the 1WT:1G58D strain

could be repressed by transient Hsp104 inhibition, demonstrating that Hsp104 plays a role in daughter-specific prion loss. In contrast, we did not observe a protection from prion loss in daughters of the 1WT:1Q24R strain with transient Hsp104 inhibition, which could indicate that daughters simply fail to receive aggregates during cell division (Fig. 3b right). However, the observation that prion loss in these strains is partially suppressed by Hsp104 heterozygous disruptions (chapter 2) suggests that Hsp104 function also contributes to Q24R-induced prion inhibition. However, as Q24R inhibits prion conversion and thus compromises the prion assembly pathway, transient inhibition of the prion disassembly pathway may not be sufficient to rescue this defect. Instead, in order to efficiently retain prion aggregates in Q24R co-expressing strains, a prolonged reduction in Hsp104-mediated disassembly might be required to achieve a proper balance with the dampened aggregate assembly pathway operating in these strains.

Based on several of my observations, more effective aggregate resolution in daughter cells would be predicted to occur if the enzyme-to-substrate ratio of Hsp104-to-prion aggregates is higher in daughter cells (Fig. 3b left). To determine if this is true, relative ratios of Hsp104 to aggregated Sup35 could be assessed by quantitative westerns in mother and daughter-enriched populations. Additionally, the efficiency of Hsp104 transmission to daughter cells during cell division could be assessed using an Hsp104-GFP fusion protein and Fluorescence Loss in Photobleaching (FLIP), as we have previously done for Sup35 (Derdowski et al. 2010). The efficiency of Hsp104 transmission can then

be compared to the relative efficiency of Sup35 aggregate transmission. Furthermore, this assay can be used to determine whether the presence and conformation of $[PSI^+]$ aggregates alters the transmission or movement of Hsp104. Overall, this work should illuminate the dynamics between the two key players in prion propagation within the population.

Heat-shock enhances prion destabilization by dominant-negative mutants

My data have emphasized the importance of aggregate thermodynamic stability and chaperone function in mediating dominant-negative prion loss. It has previously been demonstrated that $[PSI^+]^{\text{Weak}}$ strains are susceptible to prion loss when heat-shocked for 45min at 40°C, a condition which induces the expression of several molecular chaperones (Werner-Washburne and Craig 1989; Sanchez and Lindquist 1990; Newnam et al. 2011). Therefore, I determined whether this condition could enhance prion loss in dominant-negative mutant co-expressing cells. Heat-shock led to a substantial enhancement of prion loss in G58D or Q24R co-expressing $[PSI^+]^{\text{Strong}}$ strains, the wildtype counterparts of which are largely insensitive to this treatment. Interestingly, the degree of prion loss induced by heat-shock in dominant-negative mutant strains correlates with their corresponding propagon numbers and thermodynamic stabilities, with strains in the lower range for both properties displaying the greatest instability.

These data demonstrate that, like prion variant thermodynamic stability, chaperones can differentially affect prion propagation at both functional extremes (Fig. 2) (Chernoff et al. 1995; Eaglestone et al. 2000; Ferreira et al. 2001; Ness et al. 2002; Grimminger et al. 2004). This effect is clearly dependent on the

unique sensitivity of the replicating prion conformation and sequence to chaperone intervention, potentially defined by both the number and the thermodynamic stability of prion aggregates within the cell. For instance $[PSI^{+}]^{\text{Weak}}$ variants are also more readily destabilized by Hsp104 overexpression and transient heat-shock than strong variants, and dominant negative co-expressing strains are highly destabilized by these same conditions (Tanaka et al. 2004; Cox et al. 2007; Disalvo et al. 2011; Newnam et al. 2011), perhaps revealing a similar pathway to prion loss. Therefore, prion complexes, which are either more abundant or moderately thermodynamically stable, may be more resistant to prion loss by elevated chaperone activity. If low prion complex numbers enhance their ability to be resolved by chaperone induction, reducing their numbers in the $[PSI^{+}]^{\text{Strong}}$ cells by titrating down the expression of Sup35 should increase prion loss during heat-shock.

Extension to the mammalian prion system

PrP polymorphisms confer genetic protection and dominant-negative interference in mammals

As previously mentioned, dominant-negative prion inhibition extends to mammalian prion diseases, and the mechanisms underlying prion protection in this system may suggest promising strategies for therapeutic interventions. For instance, individuals with PrP polymorphisms are genetically less susceptible to prion disease (Dickinson et al. 1968; Dickenson and Outram 1988; Maciulis et al. 1992; Laplanche et al. 1994; Belt et al. 1995; Hunter et al. 1997; Goldmann et al. 1998; Shibuya et al. 1998; Bossers et al. 2000; Hizume et al. 2009), and certain

polymorphisms can also function as dominant inhibitors of prion propagation *in vivo* (Kaneko et al. 1997; Zulianello et al. 2000; Perrier et al. 2002; Crozet et al. 2004; Atarashi et al. 2006; Furuya et al. 2006; Ott et al. 2008; Toupet et al. 2008; Hizume et al. 2009). Interestingly, our studies on the yeast dominant-negative mutants G58D and Q24R may illuminate the mechanisms of prion protection in the mammalian system as well, particularly for two intriguing PrP sequence variants, Q219K and Q172R, which display dominant-negative inhibition of wildtype PrP (Kaneko et al. 1997; Perrier et al. 2002; Hizume et al. 2009).

Mechanisms of fungal prion inhibition may extend to the mammalian prion system

The expression of dominant-negative PrP mutants Q219K and Q172R have been linked to defects in propagation of the prion state (Kaneko et al. 1997; Perrier et al. 2002; Hizume et al. 2009). Excitingly, these mutants appear to hold potential for protecting against or even reversing prion disease post-infection, suggesting that they can inhibit or resolve prion complexes that have already established themselves *in vivo* (Crozet et al. 2004; Furuya et al. 2006; Toupet et al. 2008). Furthermore, understanding the mechanism of prion inhibition by these mutants may suggest other methods that could be effective therapies for prion, or protein misfolding diseases.

However, the mechanisms of mammalian prion dominant-negative inhibition remain mainly unresolved. While early models suggested that inhibition resulted from a differential affinity of PrP sequence variants for a host-encoded cofactor (protein X) required for the conversion reaction (Telling et al. 1995); it was later

found that dominant-negative PrP mutants retain their activities in cell-free systems, suggesting that their effects are mediated directly through prion-prion interactions (Horiuchi et al. 2000; Lee et al. 2007; Geoghegan et al. 2009). Therefore, it has been suggested that these mutants interact with the templating surface of an aggregate to slow, as has been proposed for Q219K, or block, as has been proposed for Q172R, conversion of wildtype PrP, depending on their affinity for one another (Lee et al. 2007; Geoghegan et al. 2009). Potentially supporting this affinity-based model, mutants differ in their effective inhibitory ratios relative to wildtype protein, and in line with conversion inhibition, Q172R appears to be highly inefficient at converting to the prion state (Priola et al. 1994; Bossers et al. 1997; Kaneko et al. 1997; Priola 1999; Bossers et al. 2000; Horiuchi et al. 2000; Lee et al. 2007; Geoghegan et al. 2009). However, studies on Q219K are inconsistent with a conversion defect, as it can convert to the prion form *in vivo* and *in vitro*, seed prion formation of wildtype PrP *in vitro*, and associate with wildtype PrP in the prion fiber at a ratio equal to its initial concentration (Lee et al. 2007; Geoghegan et al. 2009; Hizume et al. 2009).

Interestingly, our studies on $[PSI^+]$ prion inhibition by G58D and Q24R have many parallels with the mammalian system and may, perhaps, provide a new framework for exploring the mechanisms of prion inhibition by Q219K and Q172R, respectively. First, as we have observed for $[PSI^+]$ prion inhibition, differences in the effective inhibitory ratios of dominant-negative mutants may also indicate that these mutants target different events in the prion propagation pathway *in vivo* (Priola et al. 1994; Kaneko et al. 1997; Priola 1999; Horiuchi et

al. 2000; Lee et al. 2007; Geoghegan et al. 2009). In this scenario, mutants that act at the templating surface would be effective at lower doses than those affecting fragmentation, which occurs along the length of the aggregate (Masel and Jansen 2000). Thus, it is plausible that Q172R asserts its anti-prion effects by decreasing prion conversion, while Q219K, which is only effective at higher ratios, may affect prion fragmentation or disassembly.

In line with this idea, PrP Q172R and Q219K demonstrate several traits which parallel those of Sup35 Q24R and G58D and thereby may function by inhibiting conversion or altering aggregate properties, respectively. For instance, Q24R, like PrP Q172R, interfered with prion propagation at sub-stoichiometric ratios while G58D, like Q219K, interfered only at higher ratios relative to wildtype protein (Bossers et al. 1997; Perrier et al. 2002; Geoghegan et al. 2009). In addition to displaying similar effective inhibitory ratios, PrP Q172R, like Sup35 Q24R, is conversion defective and is incompatible with prion variants of different thermodynamic stabilities (Bossers et al. 1997; Safar et al. 1998; Atarashi et al. 2006; Geoghegan et al. 2009), while PrP Q219K, like Sup35 G58D, converts to the prion conformer efficiently, destabilizes aggregates and is incompatible only with prion variants of reduced thermodynamic stability (Safar et al. 1998; Atarashi et al. 2006; Lee et al. 2007; Geoghegan et al. 2009; Hizume et al. 2009). Furthermore, like G58D in the $[PSI^+]^{\text{Weak}}$ or $[PSI^+]^{\text{Sc37}}$ variant background, expression of Q219K *in vivo* can lead to prion disease, and in some instances produces detectable amounts of protease resistant PrP (Lee et al. 2007; Hizume et al. 2009). Thus, instead of acting to decrease prion conversion, these

collective observations may be more consistent with a model by which Q219K targets a distinct event in the prion propagation pathway, such as aggregate disassembly. Interestingly, like Q24R-G58D double mutant expression in $[PSI^+]^{\text{Weak}}$, a triple dominant-negative mutant, Q171R-Q214I-Q219K, has been shown to be less effective at prion inhibition in cell culture than Q219K alone, highlighting that different outcomes can manifest through the combined effects of mutants on prion conformers (Zulianello et al. 2000). Thus, despite the distinct cellular environments in which mammalian and yeast prions propagate, the pathways of dominant inhibition that we have uncovered for Q24R and G58D may be applicable to Q172R and Q219K, respectively. These observations suggest that a similar competition between aggregate assembly and disassembly pathways occurs in the mammalian prion system, with the kinetics of these pathways modulating the metastability and phenotypic outcome of mammalian prion disease.

Prion clearance in mammals

Additionally, we may be able to further extend the insights gained from our studies on G58D inhibition to help explain the differential severities of certain mammalian prion variants. For example, the drowsy (DY) variant of hamster prion assembles into aggregates of lower thermodynamic stability than most other prion variants (Bessen and Marsh 1992; Bessen and Marsh 1992; Bessen and Marsh 1994), but it displays significantly milder disease characteristics (i.e. longer incubation period) (Safar et al. 1998; Legname et al. 2006; Tanaka et al. 2006). This observation is initially surprising, as more thermodynamically stable

and larger prion aggregates have been linked to phenotypically less severe prion states for mouse PrP, as well as for yeast $[PSI^+]$ variants. Mechanistically this link may be explained by the inefficient fragmentation of more stable aggregates and the less efficient transmission of large prion complexes *in vivo* (Fig. 1, Bottom) (Derdowski et al. 2010). However, our results with G58D co-expression in $[PSI^+]^{\text{Strong}}$ cells suggest that aggregate disassembly can also lead to prion loss if it outpaces the rate of aggregate assembly (Fig. 2a). In line with this idea, unstable hamster prion variants appear to be less efficient in promoting PrP conversion to the prion conformer and are potentially more efficiently cleared *in vivo* (Bessen et al. 1995; Mulcahy and Bessen 2004; Shikiya et al. 2010; Ayers et al. 2011).

Consistent with the idea that aggregate assembly and disassembly pathways compete with one another *in vivo*, infectious particles delivered by direct inoculation, which presumably do not represent a load on the system, are largely cleared from experimental organisms (Dickinson et al. 1975; Outram 1976; Bueler et al. 1993; Race et al. 2001; Safar et al. 2005; Sandberg et al. 2011), and even established prion infections are reversed *in vivo* upon repression of new PrP synthesis (Mallucci et al. 2003; Safar et al. 2005; Pfeifer et al. 2006; Mallucci et al. 2007; White et al. 2008). Thus, our studies of G58D yeast prion inhibition lend credence to the idea that mammalian prion variants of lower thermodynamic stability may be more effectively cleared *in vivo*.

Cellular conditions and mammalian prion disease

Chaperones might also mediate mammalian prion propagation and disease, perhaps acting to fragment aggregates and/or repress or resolve protein aggregation in an analogous manner to Hsp104 (and other chaperones) in the yeast prion system. Correlative studies on chaperone levels and prion-associated cell changes in mammalian models suggest that this may be the case. However, the effects of chaperones on prion disease and aggregation in the mammalian system remain ambiguous, as altered chaperone levels have been correlated both to enhanced and reduced prion-associated neural changes. For instance an increase in chaperone levels, particularly Hsp70 family members, have been observed during prion infection in different prion disease models (Laszlo et al. 1992; Diedrich et al. 1993; Kenward et al. 1994; Booth et al. 2004; Sawiris et al. 2007). Thus, the presence of prion aggregates in the mammalian system may act as a stress condition to induce the expression of molecular chaperones. On the other hand in a cell culture model, it has been observed that prion infection inhibits the induction of Hsp70 family members by heat-shock (Tatzelt et al. 1995). Furthermore, a negative association has been observed between prion protein deposition and HSP gene expression in some studies (Kovacs et al. 2001; Serrano et al. 2011).

Although it is possible that changes in chaperone levels are simply secondary effects of prion infection, the prospect that they may be playing a role in prion propagation remains intriguing. It is interesting to speculate that they may differentially regulate prion propagation analogously to chaperones in the yeast prion system; potentially enhancing prion infection or conversely, aiding in

protection against prions by mediating aggregate clearance (Fig. 2). Prion variants display differential distributions and severities of pathological changes in the brain (Fraser and Dickinson 1973; Fraser 1976; Dickinson and Taylor 1978; Kimberlin and Walker 1978; Carp et al. 1984; Bruce et al. 1989; Bruce et al. 1991), and it remains possible that these effects may also be mediated by the location of other cellular factors such as chaperones. In this regard, extremely high or low chaperone function, coupled with the specific thermodynamic stability of the replicating prion variant, could lead to prion loss through resolution or inefficient aggregate amplification, respectively.

Prion sequence requirements and species barriers

Altogether, the prion phenotype and its associated metastability is the outcome of a complex interplay between the prion sequence, conformation, and the cellular environment in which it resides. Thus, in addition to highlighting the importance of *trans* factor effects in the prion biogenesis pathway, our studies with G58D and Q24R add to our understanding of *cis* element requirements for prion propagation *in vivo*. While the complete prion forming domain is crucial for converting Sup35 into the prion state (Ter-Avanesyan et al. 1994; Krishnan and Lindquist 2005), it contains two distinguishable elements, the glutamine asparagine rich tract (QNR), and the oligopeptide repeat region (OPR) (Kikuchi et al. 1988; Wilson and Culbertson 1988), which each provide important, but functionally distinct, contributions to prion propagation.

Our studies augment our growing understanding of the roles played by the QNR and OPR regions respectively in establishing and maintaining the prion

conformer. Collectively, these studies suggest that the QNR tract predominantly directs homotypic interactions to induce aggregation, while the OPR influences the inheritance of these aggregates, presumably by controlling the efficiency of fragmentation (DePace et al. 1998; Liu and Lindquist 1999; Santoso et al. 2000; Parham et al. 2001; Osherovich et al. 2004; Krishnan and Lindquist 2005; Tessier and Lindquist 2007; Toyama et al. 2007). This model is consistent with our finding that a critical mutation in the QNR tract inhibited the efficiency of prion conversion, while a critical mutation in the OPR region altered prion frangibility and fragmentation.

As the initial aggregation and the subsequent ability of aggregates to divide and transmit in a balanced fashion are essential for prion maintenance, proteins must contain sequences which endow it with both of these properties to exist as stable prion conformers. Thus, although several yeast proteins contain QNR tracts, and are able to aggregate, they do not all appear to be prionogenic (Sondheimer and Lindquist 2000; Derkatch et al. 2001; Osherovich and Weissman 2001). However, sequence elements, which differ from the QNR and OPR regions of Sup35, may mediate aggregation and transmission for other prion proteins. For instance, Ure2p lacks an OPR, while PrP contains an OPR but lacks a QNR tract (Oesch et al. 1985; Masison and Wickner 1995). From our work with G58D and Q24R, it is interesting to speculate that the extent of a protein sequence to mediate the conversion and frangibility of the prion conformer may correlate to the severity and persistence of its associated phenotype.

Interestingly, the variant-specific effects displayed by G58D and Q24R are reminiscent of the species barrier and may provide additional insight into the *in vivo* requirements of cross species prion transmission. First, heterotypic prion sequences may only be capable of co-aggregation if their spectrums of accessible conformations overlap (Collinge and Clarke 2007). However, while interspecies prion transmission is dependent on the ability of a particular prion conformation to convert a heterologous sequence (Chernoff et al. 2000; Kushnirov et al. 2000; Santoso et al. 2000; Chien and Weissman 2001; Nakayashiki et al. 2001; Chien et al. 2003; Vanik et al. 2004; Jones and Surewicz 2005), our studies further demonstrate that faithful prion replication *in vivo* is also highly dependent on the properties of the final prion complex created by this co-aggregation. For instance, despite the ability of closely related Sup35 sequences to co-aggregate, a barrier to efficient prion propagation occurs among some sequence/variant combinations (Chen et al. 2007; Chen et al. 2010).

Interestingly, the QNR tract and OPR region have been implicated in mediating the barrier between divergent and more closely related species, respectively (Santoso et al. 2000; Chien and Weissman 2001; Chen et al. 2010), suggesting that the QNR might restrict the initial co-aggregation between species (Santoso et al. 2000; Chien and Weissman 2001), while the OPR region may influence the heritability of prion aggregates (Chen et al. 2010). As G58D is capable of co-aggregating with wildtype Sup35 but displays a differential effect on the replication of distinct prion variants, it may serve as an example for the role of the OPR region in mediating the formation of heritable aggregates, potentially by

determining their thermodynamic properties. Therefore, the properties of the resulting prion conformer, and not simply its ability to convert into the aggregated state, may determine its cross-species transmissibility *in vivo*. Interestingly, co-aggregation of heterologous prion sequences could result in a number of outcomes dependent on the conformation of the initial seed and the subsequent properties of the mixed complex, which may include dominant-negative interference, inefficient or successful interspecies prion transmission, altered prion phenotypes, and even the emergence of new prion variants (Kimberlin et al. 1989; Scott et al. 1989; Prusiner et al. 1990; Scott et al. 1992; Prusiner et al. 1993; Telling et al. 1994; Telling et al. 1995; Korth et al. 2003; Afanasieva et al. 2011).

Due to the differential effects of [*PSI*⁺] prion mutants, the link between their mechanism of action with their location within the prion domain, and the tractability of the yeast model system, investigating additional mutations, which differentially affect [*PSI*⁺] prion conformers, may enhance our understanding of sequence/conformer incompatibility mechanisms. For instance, sequences which compromise the conformational conversion of the prion complex may promiscuously inhibit several prion conformations, while those that are efficient at prion conversion but affect the frangibility of the prion conformation, may display differential effects strongly dependent on the properties of the replicating or infectiously introduced prion variant.

To further probe the relationship between the phenotypic outcome of sequence and conformational variant combinations with the properties of prion

conformational replication, one could attempt to isolate additional Sup35 mutants, which display variant-specific effects and investigate their mechanisms of action. As conversional mutants would be predicted to more severely compromise the $[PSI^+]$ phenotype and the propagation of the prion conformer (presumably for all prion variants) at substoichiometric ratios, dominant-negative mutations isolated in a mutagenesis screen of $[PSI^+]^{\text{Strong}}$ have all previously localized to the QNR tract (DePace et al. 1998). Thus, in order to perform a sensitized screen to target mutants that may be differentially altering the properties of the prion complex, a screen could be performed in diploid $[PSI^+]$ strains, replicating the weak or strong conformer, expressing only one endogenous copy of wildtype Sup35, and utilizing a mutant library expressed at levels modestly higher than that of the endogenous wildtype copy. Furthermore, as the OPR region appears to mediate prion complex frangibility, in order to enrich for mutants that specifically affect this property, one could mutagenize only the OPR region for a screen. The results of this screen may provide further insight into the roles of prion domain elements, as well as enhance our understanding of the differences between the properties of weak and strong prion conformers, especially if mutants which differentially affect both variants are identified.

Conclusions

Collectively, our studies on the yeast prion $[PSI^+]$ and its dominant-negative inhibition have begun to mechanistically link the conformational properties of prion propagation to its biological consequences *in vivo*. This work has enhanced

our understanding of how dynamic processes within the prion biogenesis cycle define the metastability of the prion state. While conformation-based phenotypes are created through the interplay of Sup35 folding, protein quality control and other aspects of yeast cell biology (Derdowski et al. 2010), imbalances in these forces lead to the dominant inhibition of prion propagation *in vivo* (Disalvo et al. 2011). Thus, prion propagation is determined by the competition between aggregate assembly and disassembly pathways *in vivo*, a balance ultimately mediated by the interplay between the cellular environment with the unique properties of the prion conformer.

Given the many parallels between prion propagation in yeast and mammals, the insight gained through these studies may provide a new framework for connecting protein misfolding pathways to disease mechanisms. Our model of dominant-negative prion inhibition in the $[PSI^+]$ system may illuminate the mechanisms of mammalian dominant-negative inhibition and the phenotypic variability of mammalian prion variants. While this work suggests that multiple processes involved in prion propagation may be targeted to alleviate prion disease progression, it also emphasizes the importance of the *in vivo* biogenesis cycle for variant-specific conformational replication. Thus, in order to predict the phenotypic outcome of specific prion alterations, we must have a global understanding of variant-specific conformational replication within potentially fluctuating *in vivo* environments.

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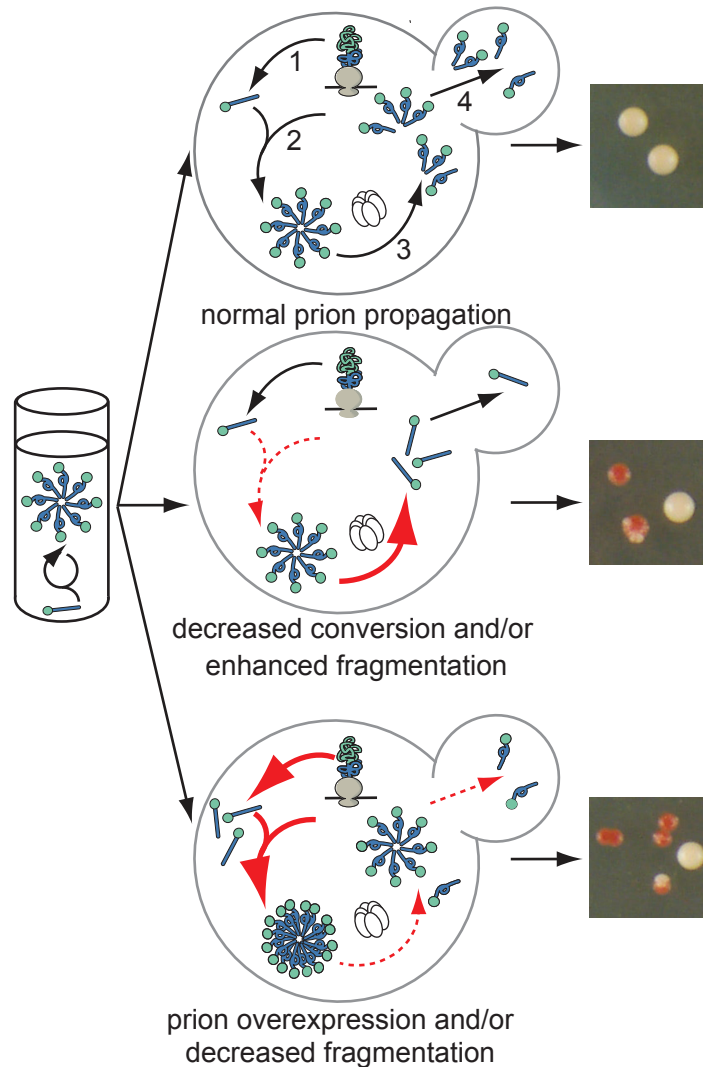


Figure 1. The cellular environment modulates prion misfolding pathways to create protein-based traits. (top) Self-replicating protein conformations create stable phenotypes (white colonies) in vivo when the processes of synthesis (1), conversion (2), fragmentation (3) by Hsp104 (hexamer) and transmission (4) are balanced to allow aggregates of prion proteins to persist in vivo. (middle) Dominant inhibition of prion propagation by mutants that decrease conversion efficiency (dotted red arrow) or enhance fragmentation efficiency (solid red arrow) promote aggregate disassembly (ball and stick) and induce prion loss (red colonies). (bottom) Overexpression of prion proteins (red arrows) or decreased fragmentation (dotted red arrows), leads to the accumulation of large aggregates that are inefficiently transmitted to daughter cells (dotted red arrow) and loss of the prion-associated phenotype (red colonies).

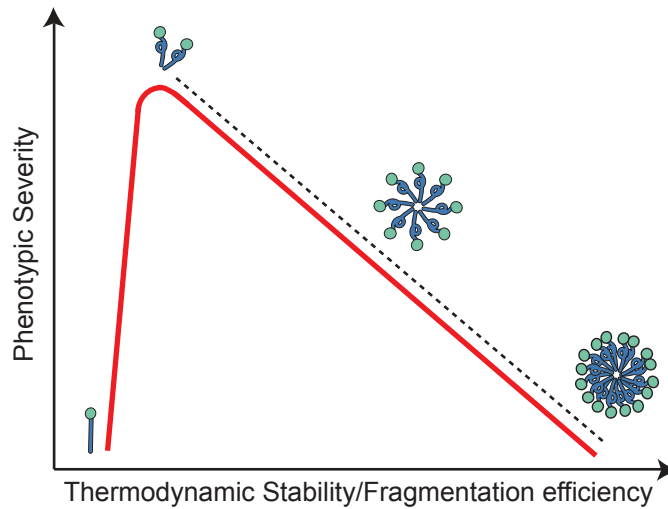


Figure 2. Relating prion phenotypic severity to aggregate thermodynamic stability. For many prion variants, there is a linear but inverse relationship between aggregate thermodynamic stability and phenotypic severity (black dotted line), but this trend cannot explain the phenotypes associated with all prion variant or the effect of dominant-negative prion mutants. Our studies in vivo on the yeast prion [*PSI*⁺] suggest that thermodynamic stability possesses a limit on prion persistence at both extremes (red line) by impacting aggregate size and accumulation (shown schematically). The least thermodynamically stable aggregates are efficiently resolubilized, while the most thermodynamically stable aggregates are inefficiently transmitted.

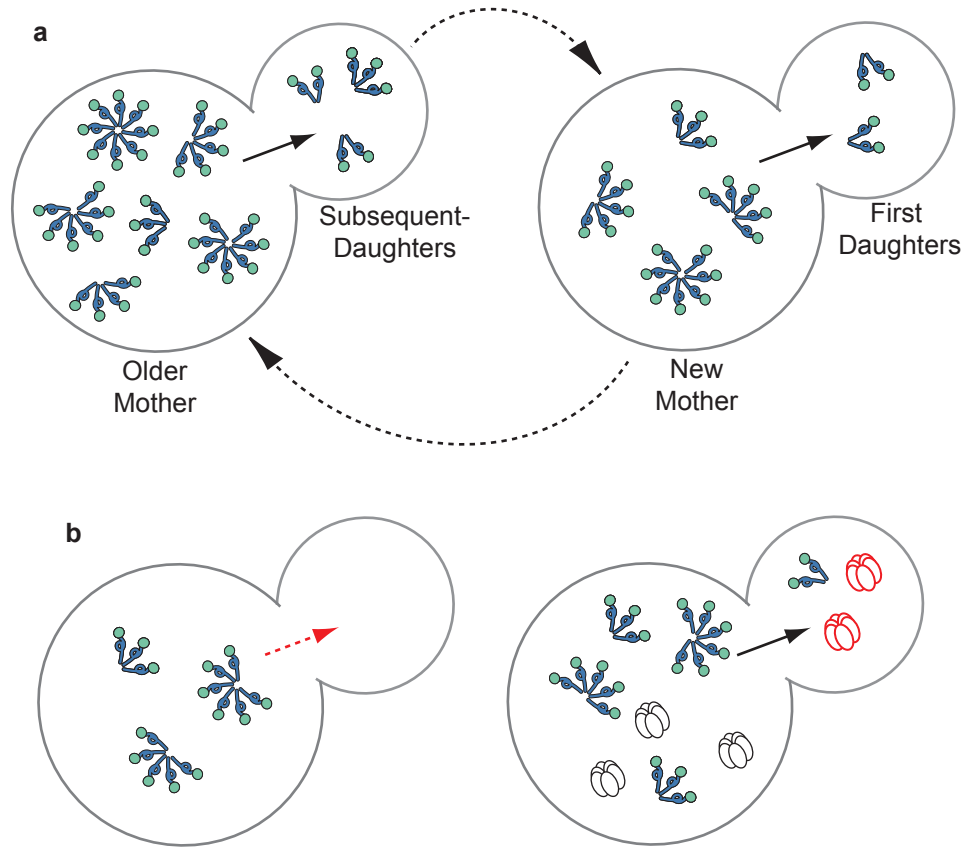


Figure 3. Population heterogeneity. a. Propagon numbers correlate to cellular age. As daughters receive a smaller percentage of prion complexes from their mothers, which take several generations to amplify, new mothers transmit fewer prion complexes to their first daughters. b. Daughter specific prion loss induced by dominant-negative mutant expression may occur in several ways, first, prion complexes may simply fail to be transmitted to daughter cells during cell division (left), or prion complexes may be more likely to undergo resolubilization in daughter cells due to differential partitioning or accumulation of chaperones and prion complexes between cells (right).

Appendix: The Impact of Population Heterogeneity on Prion Loss

I performed all the experiments described in the appendix

Introduction

Prions are self-propagating protein aggregates that give rise to unique phenotypic states. A set of severe neurodegenerative disorders in mammals termed Transmissible Spongiform Encephalopathies (TSEs) are associated with the self-replicating misfolded and aggregated state of the prion PrP (Griffith 1967; Prusiner 1982), while several proteins can assemble into self-propagating prion aggregates which confer metastable traits in fungi (Wickner 1994; Tuite and Serio 2010). Typically, these phenotypes are the result of a change in the physical state of the protein; with the soluble and monomeric (non-prion) form having a different function than the aggregated (prion) form (Tuite and Serio 2010).

One fascinating aspect of prion biology is that the proteins do not simply confer a binary phenotype, but create instead a spectrum of biological outcomes (Bessen and Marsh 1994; Derkatch et al. 1996; Safar et al. 1998; Schlumpberger et al. 2001; Bradley et al. 2002; Wickner et al. 2009). This phenotypic continuum results from the conformational flexibility of the prion protein, which allows it to assemble into aggregates with unique physical properties, referred to as prion variants (Bessen and Marsh 1994; Telling et al. 1996; Caughey et al. 1998; Safar et al. 1998; Peretz et al. 2001; Peretz et al. 2002; Tanaka et al. 2004; Tanaka et al. 2006; Toyama et al. 2007). But, it is ultimately the cellular environment in which these prion aggregates must replicate that mediates the stability of the prion variant and determines the phenotypic readout (Derdowski et al. 2010; Li et al. 2010; Disalvo et al. 2011). Nonetheless, the complex interplay between prion

aggregates with their cellular environment is just beginning to be elucidated for mammalian and fungal prions. Due to its ability to be easily manipulated *in vivo*, the yeast prion Sup35 serves as an invaluable system with which to probe the relationship between the prion conformation with its cellular environment.

Sup35 functions as an essential release factor that aids in translation termination (Stansfield et al. 1995; Zhouravleva et al. 1995). In the aggregated prion forms ($[PSI^+]$), the function of Sup35 is compromised to varying degrees based on the specific conformation adopted, while in the non-prion soluble form ($[psi^-]$), Sup35 efficiently terminates translation (Cox 1965; Patino et al. 1996; Paushkin et al. 1996). $[PSI^+]$ states are metastable (Cox et al. 1980; Lund and Cox 1981), and the frequency of prion loss is impacted by Sup35 conformation, which alters the biogenesis of prion aggregates *in vivo*. For instance, Sup35 prion conformers which assemble into larger and more thermodynamically stable aggregates do not accumulate or transmit as efficiently as less thermodynamically stable aggregates (Kryndushkin et al. 2003; Tanaka et al. 2006; Pezza et al. 2009; Derdowski et al. 2010).

The metastability of prion phenotypes is likely to result from variations in both aggregate size and number. For example, the size of prion aggregates inversely correlates with mitotic stability (Derkatch et al. 96, Kryndushkin et al. 03), and overexpression of Sup35, which increases aggregate size, decreases mitotic stability (Allen et al. 2005; Derdowski et al. 2010). Moreover, Hsp104 activity determines the size, and thereby the transmission, of prion aggregates and, when present at higher levels, can alleviate the mitotic instability of variants or

conditions which produce large prion complexes (Borchsenius et al. 2001; Borchsenius et al. 2006). In contrast, conditions which create highly thermodynamically unstable, smaller, and fewer prion complexes, such as the co-expression of dominant-negative mutations, lead to prion loss, which is exacerbated by increased Hsp104 levels (Disalvo et al. 2011). Additionally, there is a positive correlation between propagon numbers and the mitotic stability of prion variants (Cox et al. 2007).

These variations in aggregate size and number, which are critical for prion stability, are also impacted by their cellular context. Propagons appear to partition differentially between mother and daughter cells, with daughters inheriting approximately only half of the propagons retained by the mother, which is proportional to the relative volumes of mother and daughters during cell division (Cox et al. 2003). This differential transmission of aggregates from one cell to another creates age-dependent variation in aggregated and soluble Sup35 and also gives rise to phenotypic heterogeneity within a population (Derdowski et al. 2010).

As differential aggregate complements have been shown to exist among cells within a population, it is also feasible that cells could differ in their concentrations of other cellular factors important for prion propagation such as molecular chaperones. Therefore, a spectrum of unique cellular subpopulations could co-exist and interchange over time, creating more complex and dynamic environments in which the prion conformer differentially replicates. While a change in the ratios between prion complexes and molecular chaperones affects

prion stability and the phenotypic readout of the prion state (Chernoff et al. 1995; Borchsenius et al. 2001; Borchsenius et al. 2006; Disalvo et al. 2011), environmental conditions, such as heat shock, can also lead to prion destabilization [(Newnam et al. 2011) reviewed in (Chernoff 2007)], presumably by altering the levels of aggregated proteins and chaperones in the cell. Thus, it is likely that certain cellular subsets may be primed for prion loss or modulation, depending on their unique aggregate to chaperone complements.

Using unstable $[PSI^+]$ yeast prion strains co-expressing wildtype and dominant-negative prion mutants, Q24R and G58D, the effect of replicative age and heat shock on prion destabilization was investigated. Daughter cells were found to be more susceptible to prion loss; and, in strains co-expressing G58D, this loss was dependent to some extent on Hsp104 function. Furthermore, heat shock exacerbated prion loss in dominant negative co-expressing cells, with the severity of the effect correlating inversely to the aggregate thermodynamic stability and the number of propagons for each strain. Together, our results highlight the interplay between cellular states and prion phenotypes, suggesting that prion aggregates with particular properties can become susceptible to resolution by modest fluctuations within the cellular environment.

Results

Relationship between replicative age and propagon accumulation

I previously demonstrated that prion seeds (propagons) increased in subsequent daughters isolated from a new mother in a $[PSI^+]^{\text{Strong}}$ strain (Derdowski et al.

2010). This trend was recapitulated in $[PSI^+]^{\text{Strong}}$ cells co-expressing an additional wildtype (Fig. 1a) or G58D mutant (Fig. 1b), demonstrating that it is a general phenomenon and also occurs in strains co-expressing a dominant-negative mutant. Thus, daughters not only receive fewer aggregates initially, but they also take several generations to amplify the aggregates that they receive.

Prion loss occurs preferentially in daughter cells

The limited number of propagons transmitted to daughters suggests that this population should be inherently more sensitive to prion loss. To test this idea, the percent of $[psi^-]$ colonies in daughters relative to the total population was compared in unstable prion strains ($[PSI^+]^{\text{Strong}}$ diploids expressing 1:1 WT to G58D or Q24R; Fig. 2). For both strains, daughters gave rise to approximately 50 percent more $[psi^-]$ colonies than the total population, suggesting that prion loss occurs preferentially in daughter cells (Fig. 2a).

Hsp104 function plays a role in daughter-specific prion loss for G58D co-expressing strains

Prion loss in daughters could occur in several ways. The simplest explanation is that prion complexes fail to transmit to daughter cells (Tanaka et al. 2006). However, an alternative possibility is that the prion complexes transmitted to daughters are more sensitive to resolubilization to Hsp104, a process that contributes to dominant-negative prion inhibition (Disalvo et al. 2011). Given that daughters receive fewer (Fig. 1) and smaller prion complexes (Derdowski et al. 2010), this population should be primed for resolution of existing aggregates. To

discriminate between these two mechanisms, lack of transmission or daughter-specific resolubilization, I determined if Hsp104 activity in daughter cells was required for prion loss. The relative percentage of [*psi*⁻] colonies isolated from daughters incubated for three hours on media containing 3mM guanidine (Gdn), which inhibits Hsp104 (Grimminger et al. 2004), was compared to the percentage of [*psi*⁻] colonies isolated from daughters separated directly onto rich media. Interestingly, daughters from the 1WT:1G58D strain incubated on Gdn for 3 hours produced significantly fewer [*psi*⁻] colonies than their non-Gdn treated counterparts (Fig. 2b), demonstrating that Hsp104 function is required for prion loss in daughter cells. For the 1WT:1Q24R strain however, Gdn incubation did not significantly alter the relative percentage of [*psi*⁻] daughter colonies (Fig. 2b). Thus, Gdn treatment is not sufficient to suppress prion loss in these daughter cells. While these observations could indicate that Hsp104 activity is not required for Q24R-mediated prion inhibition, the effects of Q24R expression are partially reversed by a heterozygous disruption of Hsp104 (chapter 2). An alternative possibility is that Hsp104 is not the limiting factor under these conditions. For example, the conversion defect of Q24R could reduce aggregate abundance so significantly that daughter cells cannot amplify the aggregates that they do inherit to a level that supports [*PSI*⁺] due to other constraints on the system, such as the cell-division cycle.

Heat shock enhances prion loss in PNM expressing strains

As certain subpopulations of cells are more susceptible to prion loss and to the function of Hsp104, it seemed plausible that environmental conditions, which

alter cellular physiology (especially molecular chaperone expression), might shift a population towards a state that is more susceptible to prion loss. Incubating yeast cultures at temperatures over 30 degrees (heat-shock) induces the expression of molecular chaperones, including Hsp104 (Sanchez and Lindquist 1990) and has previously been shown to destabilize a $[PSI^+]^{Weak}$ variant (Newnam et al. 2011). Because a 40°C heat shock is sufficient to induce prion loss in a $[PSI^+]^{Weak}$ but not a $[PSI^+]^{Strong}$ strain (Newnam et al. 2011), its phenotypic effect on $[PSI^+]^{Strong}$ diploids expressing wildtype and G58D or Q24R was examined. Consistent with previous observations, 1WT:1WT $[PSI^+]^{Strong}$ strains showed no phenotypic instability after a 45 or a 180-minute heat-shock, producing all white colonies at each time point (Fig. 3). In contrast, the percent of $[psi^-]$ and sectorized colonies increased for the mutant expressing cells (Fig. 3), demonstrating that prions replicating in this population are sensitive to loss upon heat-shock. The increase in $[psi^-]$ colonies at 45 and 180 minutes in the 1WT:1G58D diploid strain post-heat-shock was statistically significant (p values of 0.002 and <0.0001, respectively) and for the 1WT:1Q24R diploid strain (p values of 0.035 and 0.045, respectively). Because prion aggregates in dominant-negative mutant expressing cells are more effectively resolved by molecular chaperones than their wildtype counterparts (chapter 2), it seems feasible that the increased expression of molecular chaperones by elevated temperatures leads to prion loss by enhancing prion resolution, an idea that can be directly tested by inhibiting new Sup35 synthesis after heat shock and monitoring the physical state of existing Sup35 by sensitivity to SDS.

Diploid 1WT:1Mutant strains have fewer propagons and more chaperones relative to Sup35 than haploid strains expressing an additional G58D or Q24R Sup35 copy (Disalvo et al. 2011). Thus, I reasoned that prion curing during heat-shock, if caused by prion resolubilization, would be reduced in haploid strains, as they have lower ratios of chaperones to prion complexes. Indeed, $[PSI^+]$ loss in a haploid strain co-expressing G58D was substantially lower than its diploid counterpart ($p=0.0037$), however, this strain still displayed a significant increase in prion loss ($p=0.019$) after 45 minutes at 40°C (Fig. 3b). Haploid strains co-expressing an additional Q24R copy also displayed elevated prion loss after 45 minutes heat-shock ($p=0.0028$ 0 to 45) (Fig. 3b). Although the Q24R-expressing strains produced on average fewer $[psi^-]$ colonies upon heat shock than their diploid counterpart, this increase was not statistically significant.

Discussion

Our studies on the cell biological basis of prion curing by dominant-negative mutants suggest an intriguing link between prion aggregate properties and abundance and their cellular environment in mediating prion stability. First, prion destabilization appears to occur preferentially in a subset of cells in the population, with the appearance of $[psi^-]$ cells preferentially occurring in the daughter population. Although this process in some instances may simply result from a lack of aggregate transmission to daughters, our studies are consistent with a loss of transmitted aggregates, perhaps reflecting different ratios of prion aggregates to chaperones in subpopulations of cells. That transient Hsp104 inhibition in daughter cells resulted in a decrease in $[psi^-]$ colonies in 1WT:1G58D

diploids suggests that Hsp104 may be more efficiently resolving aggregates in these cells. Whether this process is specific to G58D expressing cells or can occur for other prion mutants remains to be tested.

The role of the cellular environment in mediating the prion state was additionally demonstrated by prion loss in mutant-expressing cells after heat-shock. Interestingly, heat-shock has been previously reported to only significantly destabilize certain prion conformational variants (Newnam et al. 2011), suggesting that specific prion conformations are more susceptible to the effects of heat-shock. At this stage, the mechanism of prion loss by heat-shock remains debatable. One proposed model suggests that Hsp104-mediated prion fragmentation, and subsequent prion aggregate transmission, is inhibited upon heat shock, a situation which might result from its overexpression in the absence of a necessary cofactor for prion fragmentation (Newnam et al. 2011). This mechanism could explain why $[PSI^+]^{\text{Weak}}$, but not $[PSI^+]^{\text{Strong}}$, is sensitive to prion loss after heat shock, as it replicates fewer aggregates that are larger and more thermodynamically stable, and thereby less frangible and less efficiently transmitted (Kryndushkin et al. 2003; Pezza et al. 2009; Derdowski et al. 2010). Thus, if this model is correct, prions replicating fewer and larger prion complexes would be expected to be lost preferentially.

However, our results demonstrate that prion complexes which are fewer and smaller, or of thermodynamic stability equal to or less than $[PSI^+]^{\text{Strong}}$, can also be effectively lost during a 40°C and a 37°C (preliminary data, not shown) heat treatment in the presence of dominant-negative mutants. Interestingly, we

observed a range of prion loss in our strains, with the highest frequency occurring for the 1WT:1G58D diploid followed, in order, by the 1WT:Q24R diploid, Q24R co-expressing haploid, and G58D co-expressing haploid. Interestingly, this trend roughly correlates to the propagon accumulation and thermodynamic stabilities of each strain; strains with few propagons (Chapter 2, Fig. 8a), which are predicted to be thermodynamically less stable (Chapter 2, Fig. 3d), are most susceptible (i.e. 1WT:1G58D diploid), while those with high propagon accumulation but less thermodynamically stable aggregates (Chapter 2, Figs. 5b and 3d) are the least susceptible (i.e. G58D co-expressing haploid). Thus, although the observation that strains replicating fewer propagons are the most susceptible to heat-shock-induced prion loss could still fit with a model whereby prion loss occurs through fragmentation or transmission inhibition, the fact that our haploid $[PSI^+]^{\text{Strong}}$ G58D co-expressing strain, which has significantly more propagons and less thermodynamically stable aggregates than its wildtype counterpart (Disalvo et al. 2011), is still destabilized by heat-shock, suggests otherwise (Disalvo et al. 2011). Therefore, our observations overall are currently more consistent with a model whereby increased expression of molecular chaperones by heat-shock can lead to the dissolution of prion complexes.

If prion loss by heat-shock is induced through aggregate resolubilization a number of parameters could affect the susceptibility of prion complexes to this process. Among others, these parameters could include complex size, thermodynamic stability, abundance, rate of amplification, and transmission

efficiency as well as the presence of other aggregated chaperone substrates in the cell. Interestingly, in the $[PSI^+]^{\text{Weak}}$ strain, daughter cells are more frequently converted to $[psi^-]$ after heat-shock (Newnam et al. 2011). Therefore, when considered in light of our combined results, it is interesting to speculate that the limiting number of complexes transmitted to daughter cells, especially in $[PSI^+]^{\text{Weak}}$ and dominant negative co-expressing strains, are more susceptible to the enhanced fragmentation arising from the elevated chaperone levels induced by heat-shock. This scenario would effectively lead to aggregate resolubilization if the abundance and properties of prion complexes (thermodynamic stability and conversion efficiencies) are below the minimum threshold required to skew the balance between the aggregate assembly and disassembly pathway toward the latter process.

Overall, these data highlight the multi-faceted nature of prion biogenesis within a cellular population. We have found that the subpopulation of yeast cells containing the fewest prion complexes are most susceptible to prion clearance under the right conditions, and a similar phenomenon may help to explain the delay in disease onset under different conditions in the mammalian prion system. For instance, the incubation period for clinical disease is longer for animals expressing lower levels of PrP or receiving lower doses of PrP^{Sc} (Prusiner et al. 1990; Tremblay et al. 1998; Thackray et al. 2002). Moreover, animals previously considered to be resistant to prion infection actually replicate the prion form (Race et al. 2001), with infectivity progressively accumulating over time in brain material, suggesting partial clearance of the original isolate followed by a slow

replication phase (Race et al. 2001). Furthermore, prion disease progression and PrP^{Sc} accumulation can be reversed upon repression of PrP expression (Mallucci et al. 2003; Safar et al. 2005; Pfeifer et al. 2006; Mallucci et al. 2007; White et al. 2008). Thus, differential cellular aggregate accumulation and clearance may underlie disease onset or sub-clinical infection in the mammalian prion system. It is also interesting to speculate that imbalances in normal PrP accumulation and turnover might precipitate spontaneous prion disease.

It is fascinating that even within an isogenic population, fluctuations in the concentrations or activities of cellular components and prion complexes can create cell-to-cell variability, which in some instances can result in prion instability. This kind of heterogeneity among prion complexes within genetically identical populations has also been observed for the mammalian prion in cell culture, with continual cellular division causing a reduction in the accumulation of PrP^{Sc} (Ghaemmaghami et al. 2007) and even allowing for the generation of new prion variants (Li et al. 2010).

Interestingly, aggregate types and abundance are clearly not the only variables that influence the biological outcome of prion propagation in the $[PSI^+]$ system. Rather, other co-factors, specifically Hsp104, may play a dramatic role as well. Thus, altered levels of critical prion-interacting factors, coupled with the unique properties of the replicating prion variant, may explain the distinct cellular tropisms and phenotypic severities displayed by mammalian prion variants (Fraser and Dickinson 1973; Fraser 1976; Dickinson and Taylor 1978; Kimberlin and Walker 1978; Carp et al. 1984; Bruce et al. 1989; Bruce et al. 1991). It is

also striking that environmental conditions, which alter the levels of cellular components and could feasibly occur under natural settings, can significantly affect prion replication. Thus, our results highlight the dynamic nature of the *in vivo* prion biogenesis pathway; suggesting that unique interactions between prion aggregates and cellular factors may occur within cellular sub-populations to produce phenotypic heterogeneity or prion destabilization.

Table 1: Plasmids

Name	Description
SB657	pRS306-P _{tetO2} Sup35
SB658	pRS306-P _{tetO2} Sup35(G58D)
SB659	pRS306-P _{tetO2} Sup35(Q24R)

Table 2: Yeast strains

Strain	Genotype	Plasmid	Figure
SY1511	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35 leu2-3,112</i>	SB657	1a, 3b
SY1512	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3,112</i>	SB658	1b, 3b
SY1513	<i>MATa [PSI⁺] ade1-14 his3Δ200 trp1-289 ura3-52::URA3::P_{tetO2}SUP35(Q24R) leu2-3,112</i>	SB659	3b
SY1646	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35 leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB657	3a
SY1648	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35(G58D) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB658	2a, 2b, 3a
SY1650	<i>MATa/α [PSI⁺] ade1-14/ ade1-14 his3Δ200/ his3Δ200 trp1-289/ trp1-289 ura3-52/ura3-52::URA3::P_{tetO2}SUP35(Q24R) leu2-3,112/ leu2-3,112 SUP35/sup35::KANMX4</i>	SB659	2a, 2b, 3a

Methods

Plasmids. All plasmids used in this study are listed in Table 1 and have been previously described (Disalvo et al. 2011).

Strains. All yeast strains are derivatives of 74-D694, are listed in Table 2, and were generated as previously described (Disalvo et al. 2011).

Propagon Counts. The number of propagons per cell in subsequent daughters was performed by micromanipulation and determined by an *in vivo* dilution, colony-based method, as previously described (Cox et al. 2003; Derdowski et al. 2010).

Daughter-specific assays. In all assays, daughters were separated by micromanipulation, and moved to rich media (1/4 YPD) for color development. For Hsp104 inhibition, daughters were first moved to minimal medium (SD-complete with 2.5mM Adenine and 4% dextrose) with 3mM guanidine for three hours before being transferred to rich media. In each case only completely red colonies were counted as [*psi*].

Heat-shock. Cultures kept in log phase for at least 24hrs were incubated at 40°C for 180 minutes. Cells were plated on rich media with 10µg/L Doxycycline after a 0, 45, and 180-minute heat-shock and incubated for several days at 30°C, to observe colony color in the absence of the continued expression of the integrated Sup35 allele. The percentage of red, white, and sectorial colonies were determined for each time point.

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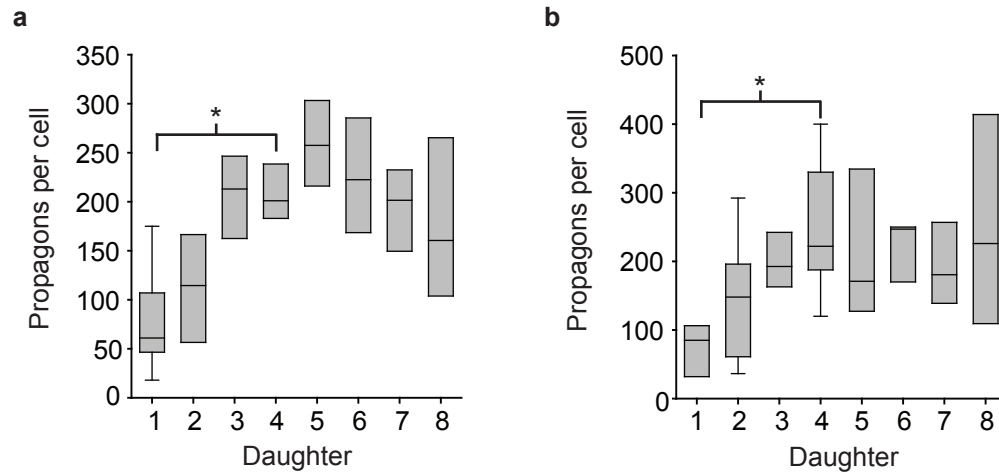


Figure 1. New daughters receive fewer prion complexes. Newly budded daughter cells from haploid wildtype yeast strain expressing WT Sup35 (a) or G58D (b) from P_{tetO2} were isolated by micromanipulation and the propagons in each were quantified. Horizontal lines on boxes indicate 25th, 50th, and 75th percentiles; error bars indicate 10th and 90th percentiles, * $p=0.0001$ (a) or * $p=0.0002$ (b).

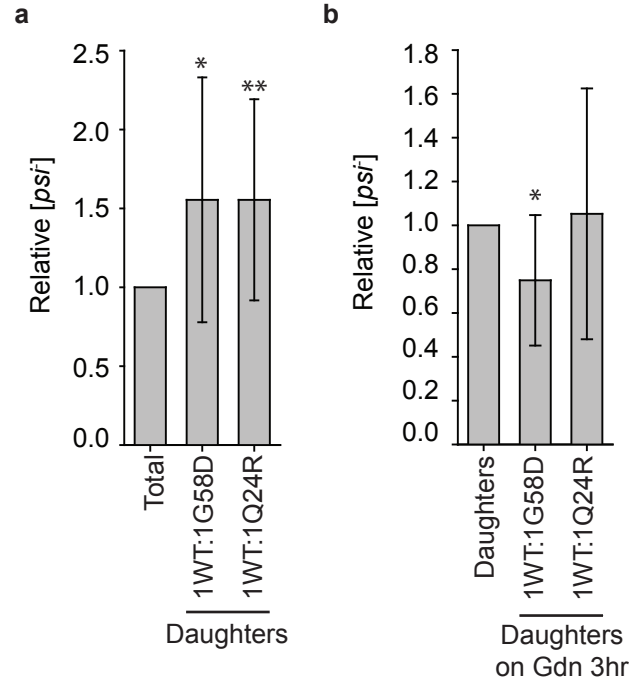


Figure 2. Prion loss occurs preferentially in daughter cells and is mediated by Hsp104 function in G58D expressing strains. **a.** Budded daughters from diploid [*PSI*⁺]^{Strong} strains expressing one endogenous WT and one WT or Mutant Sup35 from P_{tetO2} were isolated via micromanipulation onto rich media and the percent of [*psi*⁺] (red) colonies were determined relative to the total population of the same culture (*p= 0.028 **p= 0.0016). **b.** The percent of [*psi*⁺] colonies from budded daughters isolated onto rich media + 3mM Gdn for three hours were determined relative to the percent [*psi*⁺] colonies from daughters from the same culture isolated directly onto rich media (*p= 0.022).

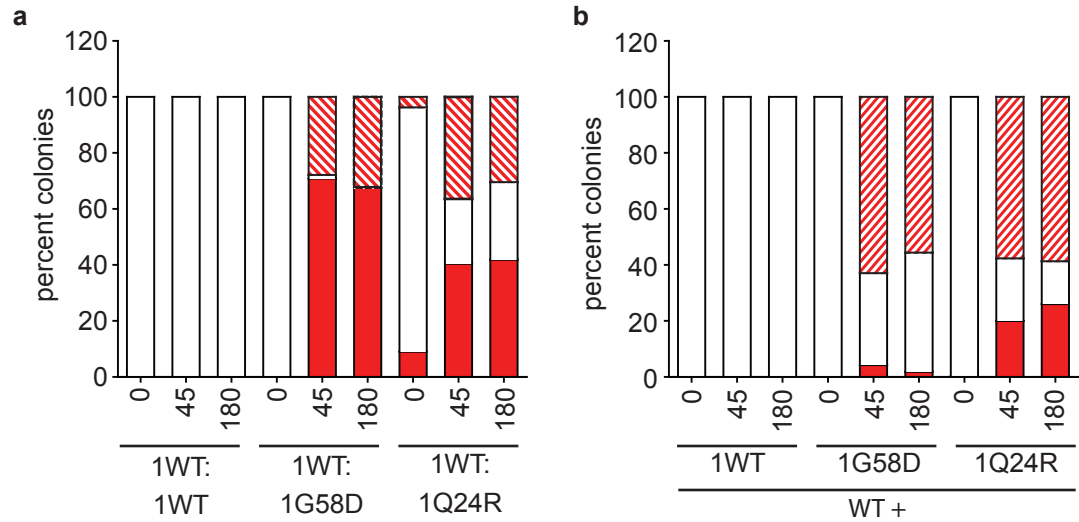


Figure 3. Heat-shock destabilizes prions in dominant-negative co-expressing cells. **a.** Cultures of diploid $[PSI^+]^{Strong}$ strains expressing 1 endogenous WT and 1 WT or Mutant Sup35 from P_{tetO2} were incubated at 40°C for the indicated times and plated onto rich media (1/4YPD) + 10μg/ml doxycycline and the number of red (red bars), white (white bars), and sectorial (red hatched bars), colonies were determined. Bars represent averages of 4 experiments. **b.** Cultures of haploid $[PSI^+]^{Strong}$ strains expressing an extra copy of WT or Mutant Sup35 from P_{tetO2} where heat-shocked as described in (a). Bars represent averages of 3 experiments.