Exploring the *de novo* Establishment of [*PSI*$^{+}$] Prion Variants and the Role of a Heterologous Prion

By

JAYA SHARMA
B.S., National College, Tribhuvan University, Nepal, 2001
M.S., Tribhuvan University,Kathmandu, Nepal, 2004

THESIS

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Defense committee:

Peter Okkema, Chair
Susan W. Liebman, Advisor
David E. Stone
Q. Tian Wang
Liming Li, Northwestern University

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LIST OF ABBREVIATIONS

Aβ	Amyloid –β-protein
DNA	Deoxyribonucleic acid
GFP	Green Fluorescent protein
GuHCl	Guanidine Hydrochloride
HSP	Heat Shock Protein
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PrP	Prion protein
PrP^{Sc}	PrP protein in Scrapie form
PCR	Polymerase chain reaction
PVDF	Polyvinyl dihydrofluoride
PrD	Prion Domain
PMSF	PhenylMethylSulfonylFluoride
Q/N rich	Glutamine/Asparagine rich
RNA	Ribonucleic acid
RRP	Rnq1 Reporter Protein
SD	Synthetic Dextrose
SDS	Sodium Dodecyl Sulfate
SDD-AGE	Semi-Denaturing Detergent Agarose Gel Electrophoresis
TSE	Transmissible Spongiform Encephalopathy
YPD	Yeast extract Peptone Dextrose

SUMMARY

Prion diseases are caused by the abnormal folding of the cellular form of the PrP protein into an amyloid self-propagating prion form, PrP^{sc}. The subsequent deposition of these PrP^{sc} aggregates in the brain is associated with several fatal neurodegenerative diseases in mammals. Interestingly, different conformations of PrP^{sc} (called prion strains), are associated with different forms of prion diseases which vary in incubation period and neuropathology

Although there is no sequence homology between any of yeast proteins and PrP^{C} , several proteins in yeast have been shown to exist either in normal soluble form or in aggregated form. Moreover some of these yeast prions have been shown to exist in different conformations which are associated with distinct phenotypes called prion variants and are analogous to mammalian prion strains. Using yeast as a model, we explored prion variant establishment and the role of preexisting prions in the appearance of heterologous prion *de novo*, two important aspects of prion biology. In the first study, we discovered a single prion protein, Sup35, could misfold into more than one conformation in a single cell during prion induction. These multiple conformers gave rise to different progeny with distinct phenotypes. We showed that such multiple variants arising from a single cell could sometimes mature into one variant in the daughter or granddaughter generations. In other cases, an unstable variant continued to propagate prions of more than one variant indefinitely.

SUMMARY (continued)

In the second study, we explored how variants of the pre-existing prion [*PIN*$^+$] differentially affect the frequency of appearance of the heterologous prion [*PSI*$^+$]. We showed that [*PIN*$^+$] variants have specific preference to cross-seed certain variants of [*PSI*$^+$] suggesting structural analogy and direct interaction between [*PIN*$^+$] seed and Sup35 protein. We showed [*PIN*$^+$] variants differ in their number of propagons. However, it was not in accordance with their [*PSI*$^+$] seeding efficiency. Additionally we showed they do not differ in the level of co-immunocaptured Rnq1 during induction of [*PSI*$^+$]. This suggests that the differential effects of [*PIN*$^+$] variants on [*PSI*$^+$] induction is specific to their conformations and comes into play following binding of Sup35 to [*PIN*$^+$] prion seed.

CHAPTER I

INTRODUCTION

I. INTRODUCTION

A. Mammalian prion

Many human diseases are associated with protein misfolding and aggregation. A group of neurodegenerative diseases called Transmissible Spongiform Encephalopathies (TSE) are caused by the conversion of the normal cellular form of the PrP protein, PrP^{C}, to the infectious and aggregated form, PrP^{Sc}. These aggregated protein entities responsible for fatal pathology are called prions (Prusiner, 1998). Prion disease affects wide range of host including scrapie in sheep, 'mad-cow disease' in cattle and chronic wasting disease in deer. The normal, cellular and alpha helical structure of PrP gets converted into an insoluble, beta sheet rich and aggregated form of PrP which forms deposits in brain tissue. Once PrP acquires the prion form, it can recruit the soluble form of the protein to join the aggregate and continue this reaction in an auto catalytic fashion (Aguzzi *et al.*, 2007; Pan, 1993).

The most common mode of prion disease transmission is sporadic where prion protein spontaneously folds in the prion form. Certain mutations predispose the PrP protein to misfold into prion form. When this occurs it is a genetic mode of transmission. Another mode of transmission is infection with a piece of the prion aggregate. Infection among the same species is very efficient whereas infection between different species is less efficient requiring a longer incubation

period and manifesting differences in the disease signs and symptoms (Collinge, 2001). This is called a ‘species barrier’. It arises because although the PrP protein, is highly conserved in mammals, the slight differences in primary sequence in different species appears to inhibit the ability of the PrP^{Sc} prion aggregate from one species to convert PrP^{C} from another species into PrP^{Sc} (Collinge, 2001; Collinge and Clarke, 2007).

B. <u>Other protein misfolding diseases</u>

Amyloid diseases are another class of neurodegenerative disease which share with prion disease the features of protein aggregation into amyloid, deposition of amyloid leading to impaired brain function, cause memory changes, dementia, and neurodegeneration. Alzheimers, Huntington’s and Parkinson’s are few examples of amyloid diseases and are caused by the deposition of Aβ, mutant Huntingtin protein and α-synuclein respectively (Soto, 2001, 2003). Amyloid diseases were thought to be different from prion in being non-infectious, however recent work and evidence suggest that Alzheimer’s disease may also be infectious but may have a very long incubation period (Barnham *et al.*, 2006; Colby and Prusiner, 2011). Such infectious amyloids have been referred to as ‘prionoids’ (Aguzzi and Rajendran, 2009). Prionoids are much more restricted in transmission than prions (Aguzzi and O'Connor, 2010).

C. Prion strains

The various forms of viral and bacterial diseases arise either from the mutation or polymorphism in the coding sequence of the genome. In contrast, the various forms of prion diseases arise from the same primary sequence of prion protein (PRNP), that acquires different conformations (Fig.1). These distinct PrP^{Sc} conformations preferentially target different regions of the brain leading to different clinical symptoms and neuropathologies. These, multiple forms of prion proteins that arise from one primary PrP sequence are called 'strains'.

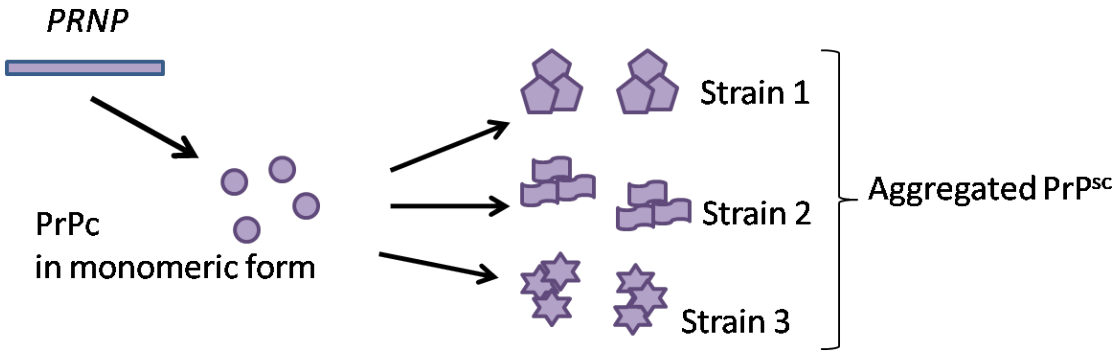

Figure 1. Multiple conformers/strains of mammalian prion, PrP^{Sc}, could arise from the same primary sequence of *PRNP*. More than one form of prion conformer could result from one primary sequence and these multiple forms target different brain regions, produce diversity of sign and symptoms and neuropathology.

D. Prions in Yeast and Fungi

Prions are not restricted to mammals, rather they have also been uncovered in lower eukaryotes like in yeast and fungi. In 1994, the prion hypothesis was modified to include other proteins that behave like prions (Cox, 1965; Wickner, 1994). These traits in yeast shared unusual 'non-Mendelian' pattern of inheritance in genetic crosses. These traits could be transmitted between the cells by cytoduction (without nuclear fusion) and expressed the associated phenotype suggesting their determinants had extra chromosomal location. Similar to mammalian prions, fungal prion protein acquire at least two structurally distinct states (monomeric and highly ordered) which differ functionally and structurally.

According to the prion paradigm, a normally folded soluble protein can occasionally misfold, and once misfolded, fungal prion proteins aggregate and propagate their abnormal state by recruiting soluble molecules of the same protein to join the aggregate.

Several prions have been well studied in yeast, *Saccharomyces cerevisiae*, those form prions which are not fatal to the host cell and confer heritable traits, namely [*PSI*$^{+}$] (Wickner *et al.*, 1995), [*PIN*$^{+}$] (Derkatch *et al.*, 2001), [*URE3*] (Wickner *et al.*, 1995), [*SWI*$^{+}$] (Du *et al.*, 2008), [*OCT*$^{+}$] (Patel *et al.*, 2009), [*MOT3*] (Alberti *et al.*, 2009) and [*GAR*$^{+}$] (Brown and Lindquist, 2009).

[HET-s] is another prion characterized in filamentous fungi *Podospora anserine (*Coustou et al., 1997*)*. Even though yeast prion and mammalian prion do not share sequence homology, they (majority of them) share the properties of being β-sheet rich, amyloid like and proteinase-K resistant. Also, both the mammalian and yeast prions are transmitted by a protein only mechanism and both of them recruit and convert soluble molecules of the protein into the prion conformation.

Mammalian prion is always associated with fatal neurodegeneration while it remains a debate whether the presence of prions in yeast provides an evolutionary advantage or is a disease. Reports that presence of certain variants of [*PSI*$^{+}$] in yeast provided the yeast with resistance to stress suggested that this could help yeast to survive stress (Tyedmers *et al.*, 2008). Recent study showed the presence of [*PSI*$^{+}$] polymers in 10 out of 690 wild type yeast strains. However, the discovery of toxic [*PSI*$^{+}$] variants suggested they might not be beneficial but represent a disease (McGlinchey *et al.*, 2011; Wickner *et al.*, 2007). Furthermore, the reports of conversion to lethal variant of [*PSI*$^{+}$] in the presence of mutated form of chaperone (i.e. Hsp40, Sis1,) suggested the potential role of chaperone to protect the cells from the toxic effects of [*PSI*$^{+}$] (Kirkland *et al.*, 2011). Also the presence of polymorphism in the sequence of *SUP35* among wild yeast is proposed to provide strong transmission barriers among them preventing the spread of [*PSI*$^{+}$] infection (Bateman and Wickner,

2012) supports the arguement. Further study is needed to clarify the role of prions in yeast.

[*PSI*$^{+}$] and [*URE3*] were known as non-Mendelian heritable elements in yeast for 30 years. During this time the molecular basis of their transmission and genetic or physical association with any extranuclear nucleic-acid species was unknown (Aigle and Lacroute, 1975; Cox, 1965). In 1994, Reed Wickner proposed that [*PSI*$^{+}$] and [*URE3*] are the prion forms of Sup35 and Ure2 proteins respectively (Wickner, 1994). In 1994, Reed Wickner proposed three genetic criteria for yeast prions.

I). Unlike bacterial plasmid, yeast mitochondrial DNA , dsRNA viruses etc. yeast prions could arise spontaneously at a low frequency even after curing because the protein capable of conversion to a self replicating prion form is always present in the cell. This is termed reversible curability. [*PSI*$^{+}$] had been shown to be cured by high osmotic media (Singh *et al.*, 1979) and guanidine hydrochloride (Tuite *et al.*, 1981) but cells cured in this way could become [*PSI*$^{+}$].

2). The higher the expression of the prion protein, the higher is the likelihood that some of the molecules will acquire the prion shape. Virus and bacteria do depend on their protein for their propagation but overproduction of any of those protein would not result in *de novo* appearance of plasmid or virus. Whereas for prion protein e.g. [*PSI*$^{+}$], appearance can be greatly increased by

the overexpression of the protein (Chernoff *et al.*, 1993). This approach provides evidence for a particular non-chromosomal element as a protein.

3) Sup35 mutants have the same phenotype as [*PSI*$^{+}$] cells. The fact that *SUP35* gene is required for [*PSI*$^{+}$] formation and yet the phenotype of [*PSI*$^{+}$] resembles the *SUP35* mutant suggest that either of these events inactivate the function of Sup35. The *SUP35* mutants retain the function of translation termination, though the accuracy is compromised leading to nonsense suppression (Crouzet and Tuite, 1987) resembling the phenotype of [*PSI*$^{+}$].

These characteristics first defined the two yeast prions, and subsequently these genetic criteria have lead to identification of several other yeast or fungal prions. None of these genetic criteria are shown to be true for mammalian prions. There is no known curing method for TSE, and hence there is no question of spontaneous generation of TSE in wild type mice. Overexpression of PrP kills mice, but their tissues were not infectious suggesting that prions were not produced (Westaway *et al.*, 1994). Mutation of *PRNP* gene that encodes PrP has no known phenotype unlike the lethal phenotype of prion disease.

[*PSI*$^{+}$] and [*URE3*] have a cellular function in their normal form and exhibit loss of that function, which causes a phenotype, when in the prion form. Rnq1, a protein of unknown function, forms prion [*PIN*$^{+}$] when prionized, and in the [*PIN*$^{+}$]

state, it enhances the frequency of de novo appearance or induction of [*PSI*$^{+}$] (Derkatch *et al.*, 1997; Derkatch *et al.*, 2000).

E. [*PSI*$^{+}$]

[*PSI*$^{+}$] trait was described as a non-chromosomal genetic-element that enhanced the efficiency of the weak nonsense suppressor, *SUQ5,* for a long time(Cox, 1965;). More recently, [*PSI*$^{+}$] was shown to be the prion form of the translation termination factor, Sup35 (Cox, 1965; Wickner *et al.*, 1995). Sup35 is an essential component of the eukaryotic translation termination factor and is homologous to the mammalian eRF3. Transient overexpression of Sup35 in a [*PIN*$^{+}$][*psi*$^{-}$] cells gives rise to [*PSI*$^{+}$] daughter cells with the nonsense suppressor phenotype.

1. Domain organization of Sup35

Sup35 is divided into three domains: N- terminal (N-domain), middle and C-terminal (C- domain) domain. The N-terminal 114 amino acid residues of Sup35 were shown to be dispensable for viability and growth, but to be necessary for the propagation of the [*PSI*$^{+}$] prion. Thus the N domain is called the prion domain (Derkatch *et al.*, 1996; Ter-Avanesyan *et al.*, 1994). The M domain is not necessary for either translation termination or prion formation, but is shown to be required for the faithful maintenance of [*PSI*$^{+}$] variants (Bradley and

Liebman, 2004). C also called the globular domain, is essential for viability and translation termination factor activity, and it works in association with another essential factor, Sup45 (Stansfield *et al.*, 1995; Ter-Avanesyan *et al.*, 1993).

2. Other properties of [*PSI*$^+$]

Transient overexpression of Sup35NM fused to green fluorescent protein (GFP) in [*PSI*$^+$] cells gives rise to large fluorescent dots in [*PSI*$^+$] cells but it remains diffuse in [*psi*$^-$] cells (Patino *et al.*, 1996). Moreover, the presence of a GFP tag on genomic *SUP35* causes the *SUP35::GFP* to form tiny dot aggregates in [*PSI*$^+$] but not [*psi*$^-$] cells (Satpute-Krishnan and Serio, 2005). This microscopic assay has been used to detect and confirm the presence of [*PSI*$^+$].

Thus, Sup35 is in an aggregated and amyloid form in [*PSI*$^+$] cells (Kimura *et al.*, 2003), whereas it is in a soluble form in [*psi*$^-$] cells. When lysates from [*PSI*$^+$] cultures are subjected to ultra high speed centrifugation, Sup35 tends to localize primarily in the pellet fraction whereas in [*psi*$^-$] cells, Sup35 is found mainly in the supernatant fraction (Patino *et al.*, 1996). These [*PSI*$^+$] aggregates break down into SDS stable sub-particles if treated with SDS at room temperature (Kryndushkin *et al.*, 2003). It was shown that the size range of subparticles varied among different variant of [*PSI*$^+$] and corresponded to the size range of ~9-50 monomers of Sup35 (Kryndushkin *et al.*, 2003).

3. [*PSI*$^{+}$] variants

While direct evidence for the conformational basis of mammalian prion strains is not established, the study of prion variants (analogous to PrP strains) in yeast has significantly advanced our understanding of strain phenomenon.

The term ‘variants’ rather than ‘strains’ is used to refer to the different forms of yeast prions, because strains in yeast already refers to different genetic backgrounds.

The [*PSI*$^{+}$] prion can exist in various conformations composed of same amino acid sequence and are associated with different phenotypes. The presence of [*PSI*$^{+}$] is monitored phenotypically by the suppression of the premature nonsense codon in the *ade1-14* allele (Chernoff *et al.*, 1995). The majority of Sup35 is aggregated in [*PSI*$^{+}$] cells, making it less available for its normal function of translation termination thus decreasing the efficiency of translation termination. As a result, premature stop codons like *ade1-14* gets read through occasionally, allowing cells to make full length Ade1, and grow in medium lacking Ade and form white colonies in rich medium like YPD. In contrast, in [*psi*$^{-}$] cells the presence of soluble and functional Sup35 causes ribosome to stop at premature stop codon at *ade1-14* mutation. Thus, truncated Ade1 is synthesized and cells are unable to grow in medium lacking Ade and accumulate red pigment in the rich medium like YPD.

[*PSI*$^{+}$] Variants differ in the level of aggregated Sup35; strong [*PSI*$^{+}$] have more aggregated and less functional or soluble Sup35 whereas weak [*PSI*$^{+}$] variants have more soluble and less aggregated Sup35 (Zhou *et al.*, 1999). Hence, variants of [*PSI*$^{+}$] can be differentiated on the basis of the level of nonsense suppression; strong [*PSI*$^{+}$] variants suppress better and are white on YPD whereas weak [*PSI*$^{+}$] variants suppress less and are pink (Derkatch *et al.*, 1996).

[*PSI*$^{+}$] variants also differ in the size of SDS-stable Sup35 polymers, strong variants have smaller sized polymers than weak [*PSI*$^{+}$] variants (Kryndushkin *et al.*, 2003). This observation is consistent with the hypothesis that strong [*PSI*$^{+}$] have more numerous but smaller sized polymers with more number of ends, thus are more efficient at recruiting soluble Sup35 at their ends and polymerizing faster compared to weak [*PSI*$^{+}$]. This results in less soluble Sup35 available for translation terminantion leading to more efficient nonsense suppression. Smaller sized oligomers in strong [*PSI*$^{+}$] suggests that they are more susceptible to fragmentation by Hsp104 and are more faithfully propagated among their progeny compared to weak [*PSI*$^{+}$] variant where [*PSI*$^{+}$] loss is more prevalent (Derkatch *et al.*, 1996).

4. Role of chaperones in [*PSI*$^+$] propagation

The role of chaperones in the yeast prion propagation and maintenance has been extensively studied. Hsp104 , was the first chaperone which was shown to be involved in the propagation of [*PSI*$^+$] prion. Overexpression of Hsp104 was shown to cure [*PSI*$^+$] (Chernoff *et al.*, 1995). Shortly afterwards, another study established deletion of *HSP104* also cures [*PSI*$^+$] suggesting its active role in the prion maintenance (Kushnirov and Ter-Avanesyan, 1998). Deletion of Hsp104 caused [*PSI*$^+$] aggregates to grow and get bigger, and eventually lead to complete loss of prion aggregates in the daughter cells suggesting Hsp104 shears prion aggregates for the even distribution among daughter cells (Kryndushkin *et al.*, 2003; Satpute-Krishnan and Serio, 2005; Wegrzyn *et al.*, 2001). GuHCl, which is shown to cure yeast prions, is suggested that it does so by inhibiting the activity of Hsp104 (Jung and Masison, 2001).

Hsp70 chaperones consists of functionally redundant Ssa1, Ssa2, Ssa3 and Ssa4 proteins that affect prion propagation. The expression of any one of these Ssa protein is sufficient for viability. Ssa1 and Ssa2 are constitutively expressed where as expression of Ssa3 and Ssa4 is stress induced. Ssa chaperones together with Hsp104 and Hsp40 assist in disaggregating and refolding misfolded proteins (Rikhvanov *et al.*, 2007). Overexpression of Ssa family of chaperones increased the rate of *de novo* induction of [*PSI*$^+$] and

antagonize curing by the overexpression of Hsp104 (Allen KD, 2005); (Krzewska and Melki, 2006; Newnam *et al.*, 1999). Moreover, excess Ssa1 and Ssa2 can cure variants of [*PSI*$^{+}$] in cells with normal levels of Sup35 and Hsp104 (Mathur *et al.*, 2009). Sis1, member of Hsp40 group of chaperone is found to be important for the propagation of [*PSI*$^{+}$], [*PIN*$^{+}$] and [URE3], all three prions (Aron *et al.*, 2007; Higurashi *et al.*, 2008).

Changing the level of these chaperones either by overexpression or deletion affect [*PSI*$^{+}$] in various ways. Thus chaperones play integral role in [*PSI*$^{+}$] propagation and maintenance.

F. [*PIN*$^{+}$]

The [*PIN*$^{+}$] prion ([***P***SI^{+}] **in**ducible) was discovered as a non-Mendelian element that enhanced the appearance of [*PSI*$^{+}$] upon overexpression of Sup35 or Sup35 prion domain, Sup35NM (Derkatch *et al.*, 1997). When [*PSI*$^{+}$] was cured with GuHCl, two class of cured yeast cells were obtained, one class could induce [*PSI*$^{+}$] on overexpression of Sup35 and another where [*PSI*$^{+}$] could not be induced even when Sup35 was overexpressed. This [*PSI*$^{+}$] inducible phenotype was called as [*PIN*$^{+}$] and was shown it could be transmitted via cytoplasm. It was also established [*PIN*$^{+}$] is required for *de novo* formation of [*PSI*$^{+}$] , but not for propagation (Derkatch *et al.*, 2000). [*PIN*$^{+}$] was cured by growth on GuHCl-

containing medium satisfying one criteria of yeast prions as proposed by Reed Wickner.

A formal demonstration of 'protein-only' nature of [*PIN*$^+$] determinant was established by showing that recombinant Rnq1 purified from *Escherichia coli* can aggregate *in vitro*, and those *in vitro* generated [*PIN*$^+$] aggregates could convert [*pin*$^-$] cells to [*PIN*$^+$] upon transformation (Patel and Liebman, 2007).

Microscopic examination can easily distinguish [*PIN*$^+$] cells from [*pin*$^-$]. Expression of *RNQ1::GFP*, in [*PIN*$^+$] cells decorates the [*PIN*$^+$] aggregates making them visible under the microscope whereas remains diffused in [*pin*$^-$] cell (Derkatch *et al.*, 2001; Sondheimer and Lindquist, 2000). Intracellular Rnq1 forms large, SDS stable, amyloid-like aggregates in [*PIN*$^+$] cells whereas Rnq1 is soluble in [*pin*$^-$] cells (Bagriantsev and Liebman, 2004). On subjecting the lysates from [*PIN*$^+$] cells to ultra high speed centrifugation, Rnq1 tends localize in the pellet fraction whereas in [*pin*$^-$] cells it localizes to soluble fraction (Bradley *et al.*, 2002).

1. [*PIN*$^+$] variants

Analogous to [*PSI*$^+$] variants, [*PIN*$^+$] prion exhibits variants and they can be distinguished by the difference in phenotype. These [*PIN*$^+$] variants are categorized on the basis of their efficiency to enhance the [*PSI*$^+$] induction *de*

novo on overexpression of Sup35 or Sup35NM. Very high [*PIN*$^+$] induces [*PSI*$^+$] with the highest frequency among the [*PIN*$^+$] variants whereas low [*PIN*$^+$] induces the least with high [*PIN*$^+$] in between (Bradley *et al.*, 2002). [*PIN*$^+$] variants can also be characterized by the amount of soluble Rnq1 they contain. High speed centrifugation analysis of Rnq1 showed the solubility of Rnq1 varies among [*PIN*$^+$] variants, Very high [*PIN*$^+$] containing the most soluble Rnq1, followed by low and high [*PIN*$^+$] (Bradley *et al.*, 2002). However the dominance of these [*PIN*$^+$] variants is determined by the level of aggregated Rnq1, thus the variant with the most aggregated Rnq1 is the dominant one. On genetic crosses, the high [*PIN*$^+$] is the most dominant variant whereas the very high [*PIN*$^+$] the least (Bradley *et al.*, 2002).

Low [*PIN*$^+$] and very high [*PIN*$^+$] are also called as spontaneous [*PIN*$^+$]s as they were discovered by the prolonged incubation of [*psi*$^-$][*pin*$^-$] cells at 4ºC. [*PIN*$^+$] variants can also be differentiated by the fluorescent pattern of Rnq1-GFP in the cytosol. On inducing Rnq1-GFP, very high and low [*PIN*$^+$] variants exhibit single dot per cell in the population whereas high [*PIN*$^+$] variant shows multiple fluorescent dots per cell (Bradley and Liebman, 2003).

2. [*PIN*$^+$] and chaperones

In contrast to [*PSI*$^+$], where overexpression and deletion of Hsp104, both lead to [*PSI*$^+$] curing, only deletion of Hsp104 cured [*PIN*$^+$] (Chernoff *et al.*, 1995; Derkatch *et al.*, 1997; Kushnirov and Ter-Avanesyan, 1998). [*PSI*$^+$] and [*PIN*$^+$] curing with deletion of Hsp104 is thought to occur through the same mechanism as decrease in Hsp104 activity results in accumulation of larger polymers.

A study established that Sis1 preferentially binds with Rnq1 in [*PIN*$^+$] cells but not with soluble Rnq1 in [*pin*$^-$] cells (Sondheimer N, 2001). Moreover, Sis1 is essential for not only propagation of [*PIN*$^+$] but also [*PSI*$^+$] and [*URE3*] (Higurashi *et al.*, 2008).

G. [*PSI*$^+$] and [*PIN*$^+$] interaction

Presence of [*PIN*$^+$] greatly facilitates the *de novo* appearance of [*PSI*$^+$] (Derkatch *et al.*, 1997; Derkatch *et al.*, 2000). To explain how presence of one prion greatly enhances the appearance of another, two mechanisms have been proposed (Osherovich and Weissman, 2001); (Derkatch *et al.*, 2001). According to the cross-seeding model, preexisting prion provides imperfect template for conversion of soluble protein to prion form during early stage of prion biogenesis (Fig. 2A).

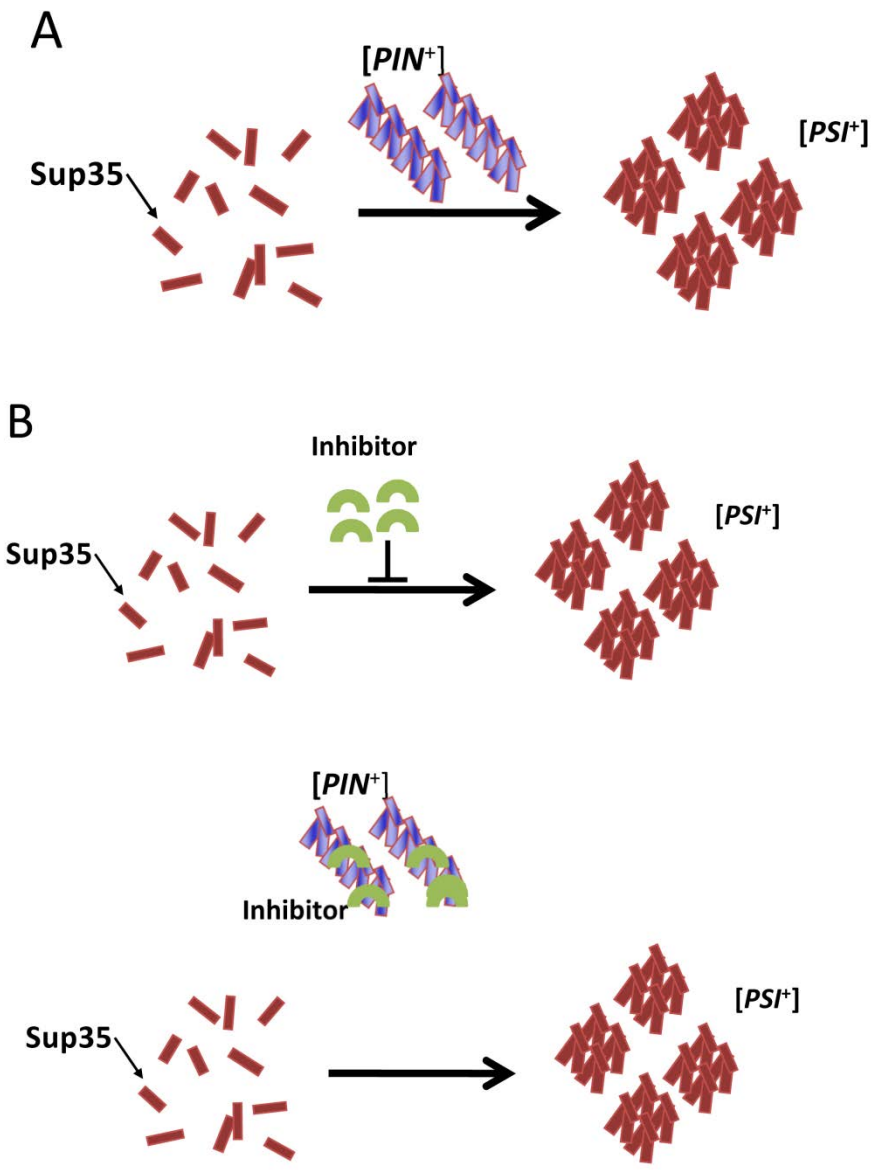

Figure 2. Cartoon depicting cross-seeding and titration model. A. Cross seeding model predicts the [*PIN*$^+$] seed directly templates the Sup35 monomers to aggregate. **B.** According to the Titration model, [*PIN*$^+$] sequesters inhibitors of de novo [*PSI*$^+$] formation enhancing the appearance of [*PSI*$^+$].

Titration model postulates that preexisting heterologous prion aggregates sequester a protein or chaperone those normally inhibit prion formation, thereby facilitating the appearance of new prion (Fig. 2B). Despite several studies done in search of titrating factors that inhibit prion formation, they have not been identified.

There are more evidences in support of cross-seeding model. *In vitro* polymerization studies done on recombinant protein Rnq1PD (amino acid residues 132-405) purified from bacteria have shown that it can form fibers *in vitro* (Glover *et al.*, 1997; Patel and Liebman, 2007). Moreover those in vitro made Rnq1PD fibers can cross-seed soluble Sup35 facilitating its polymerization and vice versa (Vitrenko *et al.*, 2007). Mutations in the prion domain of Rnq1 prion domain have been isolated which is shown to reduce the rate of *do novo* induction of [*PSI*$^{+}$] on overexpression of Sup35NM compared to wild type Rnq1 suggesting the possible interaction mediated through those residues (Bardill and True, 2009).

Microscopic studies suggest newly appearing [*PSI*$^{+}$] aggregates (following overexpression of Sup35NM and Rnq1), partially or completely colocalize with preexisting Rnq1 aggregates during *de novo* induction of [*PSI*$^{+}$], and colocalization could frequently be seen even after [*PSI*$^{+}$] establishment (Derkatch *et al.*, 2004).

Presence of [*PIN*$^{+}$] do not always positively regulate the appearance of [*PSI*$^{+}$]. There are certain variants of [*PIN*$^{+}$] which destabilize weak variant of [*PSI*$^{+}$] (Bradley and Liebman, 2003). [*PIN*$^{+}$] variants can be differentiated on the basis of Rnq1-GFP staining pattern; single dot and multiple dot variant. In the presence of multiple dot [*PIN*$^{+}$] variant, strong and weak [*PSI*$^{+}$] variants are induced whereas in the cells containing single dot [*PIN*$^{+}$], strong and unstable [*PSI*$^{+}$] are induced but not weak [*PSI*$^{+}$] variant (Bradley and Liebman, 2003).

H. <u>Amyloid growth</u>

Several studies have established that amyloid growth starts with nucleus formation. The time required for the sufficient number of nuclei to form in any particular reaction constitutes the ‘lag phase’ and is rate limiting. After enough active nuclei are formed, those nuclei template the soluble protein at their active ends thus starting the fibril growth exponentially and this constitutes the ‘log phase’. Addition of preformed amyloids/fibers to the reaction can reduce the length of lag phase or even eliminate as they provide the active nucleus to template the soluble protein and nucleation is no longer required (Serio TR, 2000). Thioflavin-T (ThT), a cationic benzothiazole dye, shows enhanced fluorescence upon binding to amyloid like oligomeric species formed either in tissue sections, cells or *in vitro* (LeVine, 1993).

I. Conclusion

PrP^{Sc}, represents the principle component of the infectious agent responsible for prion disease in mammals. Despite extensive studies to understand the mechanism surrounding the conversion of PrP^{C} to its abnormal isoforms PrP^{Sc} , how different prion strains arise from the same primary sequence and how they lead to distinct pathogenesis remains unknown. In addition to PrP, the misfolding and aggregation of a variety of other proteins into amyloid-like aggregates is also associated with more common forms of neurodegenerative diseases in humans, for e.g., Alzheimer's, Parkinson's, Huntington's etc. These diseases share the common features of protein aggregation and associated toxicity. These amyloid diseases arise with the spontaneous appearance of the first seed without known external infection. Understanding the role of preexisting heterologous seed in the *de novo* appearance of a new prion at a molecular level is very important for developing diagnostic tests and therapeutics.

Using yeast as a model system, in chapter 2, we show that different variants of [*PSI*$^{+}$] can arise from a single cell and these multiple variants generally segregate out during mitotic growth. In addition, we discovered a novel variant of [*PSI*$^{+}$] which maintained the ability to give rise to multiple variants

indefinitely. In Chapter 3, we explored the role of different variants of the preexisting prion [*PIN*$^{+}$] in the *de novo* appearance of [*PSI*$^{+}$].

J. References

Aguzzi, A., Heikenwalder, M., and Polymenidou, M. (2007) Insights into prion strains and neurotoxicity. *Nat Rev Mol Cell Biol* **8**: 552-561.

Aguzzi, A., and Rajendran, L. (2009) The transcellular spread of cytosolic amyloids, prions, and prionoids. *Neuron* **64**: 783-790.

Aguzzi, A., and O'Connor, T. (2010) Protein aggregation diseases: pathogenicity and therapeutic perspectives. *Nat Rev Drug Discov* **9**: 237-248.

Aigle, M., and Lacroute, F. (1975) Genetical aspects of [URE3], a non-mitochondrial, cytoplasmically inherited mutation in yeast. *Mol Gen Genet* **136**: 327-335.

Alberti, S., Halfmann, R., King, O., Kapila, A., and Lindquist, S. (2009) A systematic survey identifies prions and illuminates sequence features of prionogenic proteins. *Cell* **137**: 146-158.

Allen KD, W.R., Chernova TA, Müller S, Newnam GP, Winslett PA, Wittich KB, Wilkinson KD, Chernoff YO (2005) Hsp70 chaperones as modulators of prion life cycle: novel effects of Ssa and Ssb on the Saccharomyces cerevisiae prion [PSI+]. *Genetics* **169**: 1227-1242.

Aron, R., Higurashi, T., Sahi, C., and Craig, E.A. (2007) J-protein co-chaperone Sis1 required for generation of [RNQ+] seeds necessary for prion propagation. *Embo J* **26**: 3794-3803.

Bagriantsev, S., and Liebman, S.W. (2004) Specificity of prion assembly in vivo. [PSI+] and [PIN+] form separate structures in yeast. *J Biol Chem* **279**: 51042-51048.

Bardill, J.P., and True, H.L. (2009) Heterologous prion interactions are altered by mutations in the prion protein Rnq1p. *J Mol Biol* **388**: 583-596.

Barnham, K.J., Cappai, R., Beyreuther, K., Masters, C.L., and Hill, A.F. (2006) Delineating common molecular mechanisms in Alzheimer's and prion diseases. *Trends Biochem Sci* **31**: 465-472.

Bateman, D.A., and Wickner, R.B. (2012) [PSI+] Prion transmission barriers protect Saccharomyces cerevisiae from infection: intraspecies 'species barriers'. *Genetics* **190**: 569-579.

Bradley, M.E., Edskes, H.K., Hong, J.Y., Wickner, R.B., and Liebman, S.W. (2002) Interactions among prions and prion "strains" in yeast. *Proc Natl Acad Sci U S A* **99 Suppl 4**: 16392-16399.

Bradley, M.E., and Liebman, S.W. (2003) Destabilizing interactions among [PSI(+)] and [PIN(+)] yeast prion variants. *Genetics* **165**: 1675-1685.

Bradley, M.E., and Liebman, S.W. (2004) The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI+] prions. *Mol Microbiol* **51**: 1649-1659.

Brown, J.C., and Lindquist, S. (2009) A heritable switch in carbon source utilization driven by an unusual yeast prion. *Genes Dev* **23**: 2320-2332.

Chernoff, Y.O., Derkach, I.L., and Inge-Vechtomov, S.G. (1993) Multicopy SUP35 gene induces de-novo appearance of psi-like factors in the yeast Saccharomyces cerevisiae. *Curr Genet* **24**: 268-270.

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G., and Liebman, S.W. (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. *Science* **268**: 880-884.

Colby, D.W., and Prusiner, S.B. (2011) De novo generation of prion strains. *Nat Rev Microbiol* **9**: 771-777.

Collinge, J. (2001) Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci* **24**: 519-550.

Collinge, J., and Clarke, A.R. (2007) A general model of prion strains and their pathogenicity. *Science* **318**: 930-936.

Coustou, V., Deleu, C., Saupe, S., and Begueret, J. (1997) The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog. *Proc Natl Acad Sci U S A* **94**: 9773-9778.

Cox, B.S. (1965) [*PSI*], a cytoplasmic suppressor of super-suppressors in yeast. *Heredity* **20**: 505-521.

Crouzet, M., and Tuite, M.F. (1987) Genetic control of translational fidelity in yeast: molecular cloning and analysis of the allosuppressor gene SAL3. *Mol Gen Genet* **210**: 581-583.

Derkatch, I.L., Chernoff, Y.O., Kushnirov, V.V., Inge-Vechtomov, S.G., and Liebman, S.W. (1996) Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae. *Genetics* **144**: 1375-1386.

Derkatch, I.L., Bradley, M.E., Zhou, P., Chernoff, Y.O., and Liebman, S.W. (1997) Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae. *Genetics* **147**: 507-519.

Derkatch, I.L., Bradley, M.E., Masse, S.V., Zadorsky, S.P., Polozkov, G.V., Inge-Vechtomov, S.G., and Liebman, S.W. (2000) Dependence and independence of [PSI(+)] and [PIN(+)]: a two-prion system in yeast? *EMBO J* **19**: 1942-1952.

Derkatch, I.L., Bradley, M.E., Hong, J.Y., and Liebman, S.W. (2001) Prions affect the appearance of other prions: the story of [PIN(+)]. *Cell* **106**: 171-182.

Derkatch, I.L., Uptain, S.M., Outeiro, T.F., Krishnan, R., Lindquist, S.L., and Liebman, S.W. (2004) Effects of Q/N-rich, polyQ, and non-polyQ amyloids

on the de novo formation of the [PSI+] prion in yeast and aggregation of Sup35 in vitro. *Proc Natl Acad Sci U S A* **101**: 12934-12939.

Du, Z., Park, K.-W., Yu, H., Fan, Q., and Li, L. (2008) Newly identified prion linked to the chromatin-remodeling factor Swi1 in Saccharomyces cerevisiae. *Nat Genet* **40**: 460-465.

Glover, J.R., Kowal, A.S., Schirmer, E.C., Patino, M.M., Liu, J.J., and Lindquist, S. (1997) Self-seeded fibers formed by Sup35, the protein determinant of [PSI+], a heritable prion-like factor of S. cerevisiae. *Cell* **89**: 811-819.

Higurashi, T., Hines, J.K., Sahi, C., Aron, R., and Craig, E.A. (2008) Specificity of the J-protein Sis1 in the propagation of 3 yeast prions. *Proc Natl Acad Sci U S A* **105**: 16596-16601.

Jung, G., and Masison, D.C. (2001) Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions. *Curr Microbiol* **43**: 7-10.

Kimura, Y., Koitabashi, S., and Fujita, T. (2003) Analysis of yeast prion aggregates with amyloid-staining compound in vivo. *Cell Struct Funct* **28**: 187-193.

Kirkland, P.A., Reidy, M., and Masison, D.C. (2011) Functions of yeast Hsp40 chaperone Sis1p dispensable for prion propagation but important for prion curing and protection from prion toxicity. *Genetics* **188**: 565-577.

Kryndushkin, D.S., Alexandrov, I.M., Ter-Avanesyan, M.D., and Kushnirov, V.V. (2003) Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *J Biol Chem* **278**: 49636-49643.

Krzewska, J., and Melki, R. (2006) Molecular chaperones and the assembly of the prion Sup35p, an in vitro study. *Embo J* **25**: 822-833.

Kushnirov, V.V., and Ter-Avanesyan, M.D. (1998) Structure and replication of yeast prions. *Cell* **94**: 13-16.

LeVine, H., 3rd (1993) Thioflavine T interaction with synthetic Alzheimer's disease beta-amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci* **2**: 404-410.

Mathur, V., Hong, J.Y., and Liebman, S.W. (2009) Ssa1 Overexpression and [PIN(+)] Variants Cure [PSI(+)] by Dilution of Aggregates. *J Mol Biol*.

McGlinchey, R.P., Kryndushkin, D., and Wickner, R.B. (2011) Suicidal [PSI+] is a lethal yeast prion. *Proc Natl Acad Sci U S A* **108**: 5337-5341.

Newnam, G.P., Wegrzyn, R.D., Lindquist, S.L., and Chernoff, Y.O. (1999) Antagonistic interactions between yeast chaperones Hsp104 and Hsp70 in prion curing. *Mol Cell Biol* **19**: 1325-1333.

Osherovich, L.Z., and Weissman, J.S. (2001) Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion. *Cell* **106**: 183-194.

Pan, K.-M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J., Cohen, F. E. & Prusiner, S. B. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A.* **90**: 10962-10966.

Patel, B.K., and Liebman, S.W. (2007) "Prion-proof" for [PIN+]: infection with in vitro-made amyloid aggregates of Rnq1p-(132-405) induces [PIN+]. *J Mol Biol* **365**: 773-782.

Patel, B.K., Gavin-Smyth, J., and Liebman, S.W. (2009) The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion. *Nat Cell Biol*.

Patino, M.M., Liu, J.J., Glover, J.R., and Lindquist, S. (1996) Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. *Science* **273**: 622-626.

Prusiner, S.B. (1998) Prions. *Proc Natl Acad Sci U S A* **95**: 13363-13383.

Rikhvanov, E.G., Romanova, N.V., and Chernoff, Y.O. (2007) Chaperone effects on prion and nonprion aggregates. *Prion* **1**: 217-222.

Satpute-Krishnan, P., and Serio, T.R. (2005) Prion protein remodelling confers an immediate phenotypic switch. *Nature* **437**: 262-265.

Serio TR, C.A., Kowal AS, Sawicki GJ, Moslehi JJ, Serpell L, Arnsdorf MF, Lindquist SL (2000) Nucleated Conformational Conversion and the Replication of Conformational Information by a Prion Determinant. *Science* **289**: 1317-1321.

Singh, A., Helms, C., and Sherman, F. (1979) Mutation of the non-Mendelian suppressor, Psi, in yeast by hypertonic media. *Proc Natl Acad Sci U S A* **76**: 1952-1956.

Sondheimer, N., and Lindquist, S. (2000) Rnq1: an epigenetic modifier of protein function in yeast. *Mol Cell* **5**: 163-172.

Sondheimer N, L.N., Craig EA, Lindquist S (2001) The role of Sis1 in the maintenance of the [RNQ+] prion. *EMBO J.* **20**: 2435-2442.

Soto, C. (2001) Protein misfolding and disease; protein refolding and therapy. *FEBS Lett* **498**: 204-207.

Soto, C. (2003) Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat Rev Neurosci* **4**: 49-60.

Stansfield, I., Jones, K.M., Kushnirov, V.V., Dagkesamanskaya, A.R., Poznyakovski, A.I., Paushkin, S.V., Nierras, C.R., Cox, B.S., Ter-Avanesyan, M.D., and Tuite, M.F. (1995) The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in Saccharomyces cerevisiae. *Embo J* **14**: 4365-4373.

Ter-Avanesyan, M.D., Kushnirov, V.V., Dagkesamanskaya, A.R., Didichenko, S.A., Chernoff, Y.O., Inge-Vechtomov, S.G., and Smirnov, V.N. (1993) Deletion analysis of the SUP35 gene of the yeast Saccharomyces cerevisiae reveals two non-overlapping functional regions in the encoded protein. *Mol Microbiol* **7**: 683-692.

Ter-Avanesyan, M.D., Dagkesamanskaya, A.R., Kushnirov, V.V., and Smirnov, V.N. (1994) The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast Saccharomyces cerevisiae. *Genetics* **137**: 671-676.

Tuite, M.F., Mundy, C.R., and Cox, B.S. (1981) Agents that cause a high frequency of genetic change from [psi+] to [psi-] in Saccharomyces cerevisiae. *Genetics* **98**: 691-711.

Tyedmers, J., Madariaga, M.L., and Lindquist, S. (2008) Prion switching in response to environmental stress. *PLoS Biol* **6**: e294.

Vitrenko, Y.A., Gracheva, E.O., Richmond, J.E., and Liebman, S.W. (2007) Visualization of aggregation of the Rnq1 prion domain and cross-seeding interactions with Sup35NM. *J Biol Chem* **282**: 1779-1787.

Wegrzyn, R.D., Bapat, K., Newnam, G.P., Zink, A.D., and Chernoff, Y.O. (2001) Mechanism of prion loss after Hsp104 inactivation in yeast. *Mol Cell Biol* **21**: 4656-4669.

Westaway, D., DeArmond, S.J., Cayetano-Canlas, J., Groth, D., Foster, D., Yang, S.L., Torchia, M., Carlson, G.A., and Prusiner, S.B. (1994) Degeneration of skeletal muscle, peripheral nerves, and the central

nervous system in transgenic mice overexpressing wild-type prion proteins. *Cell* **76**: 117-129.

Wickner, R.B. (1994) [URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae. *Science* **264**: 566-569.

Wickner, R.B., Masison, D.C., and Edskes, H.K. (1995) [PSI] and [URE3] as yeast prions. *Yeast* **11**: 1671-1685.

Wickner, R.B., Edskes, H.K., Shewmaker, F., and Nakayashiki, T. (2007) Prions of fungi: inherited structures and biological roles. *Nat Rev Microbiol* **5**: 611-618.

Zhou, P., Derkatch, I.L., Uptain, S.M., Patino, M.M., Lindquist, S., and Liebman, S.W. (1999) The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. *EMBO J* **18**: 1182-1191.

CHAPTER II

[*PSI*$^{+}$] Prion Variant Establishment in Yeast

Jaya Sharma and Susan W. Liebman

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A. Abstract

Differences in the clinical pathology of mammalian prion diseases reflect distinct heritable conformations of aggregated PrP proteins, called prion strains. Here, using the yeast [*PSI*$^{+}$] prion, we examine the *de novo* establishment of prion strains (called variants in yeast). The [*PSI*$^{+}$] prion protein, Sup35, is efficiently induced to take on numerous prion variant conformations following transient overexpression of Sup35 in the presence of another prion, e.g. [*PIN*$^{+}$]. One hypothesis is that the first [*PSI*$^{+}$] prion seed to arise in a cell causes propagation of only that seed's variant, but that different variants could be initiated in different cells. However, we now show that even within a single cell, Sup35 retains the potential to fold into more than one variant type. When individual cells segregating different [*PSI*$^{+}$] variants were followed in pedigrees, establishment of a single variant phenotype generally occurred in daughters, granddaughters or great granddaughters—but in 5% of the pedigrees cells continued to segregate multiple variants indefinitely. The data is consistent with the idea that many newly formed prions go through a maturation phase before they reach a single specific variant conformation. These findings may be relevant to mammalian PrP prion strain establishment and adaptation.

B. Introduction

The mammalian prion is a misfolded infectious form of the PrP protein, which when accumulated in the central nervous system, leads to neurodegenerative disease (Prusiner, 1998). These include Creutzfield-Jacob disease in humans, scrapie in sheep and bovine spongiform encephalopathy in cattle. Pathogenicity is attributed to the conversion of the alpha helical rich cellular prion protein, PrP^{C}, into a beta sheet rich prion form, PrP^{Sc}, which is aggregated and protease K resistant. Differences in the conformations of infectious PrP^{sc} (that are each composed of the same PrP polypeptide) are proposed to be responsible for the distinct pathologies of prion strains (Safar *et al.*, 1998).

Several proteins in the yeast *Saccharomyces cerevisiae* have been shown to be able to form prions and many of these have been shown to confer specific heritable phenotypes (Alberti *et al.*, 2009; Crow and Li, 2011; Derkatch *et al.*, 2001; Du *et al.*, 2008; Patel *et al.*, 2009; Sondheimer and Lindquist, 2000; Suzuki *et al.*, 2012; Wickner, 1994; Wickner *et al.*, 1995). [*PSI*$^{+}$], [*PIN*$^{+}$] and [*URE*3], the prion forms of Sup35, Rnq1 and Ure2 respectively, are the best studied prions in yeast (Derkatch *et al.*, 1997; Tuite and Cox, 2003; Wickner, 1994). Similar to their mammalian counterparts, the prion forms of these yeast proteins are amyloid-like (Glover *et al.*, 1997; King *et al.*, 1997; Sondheimer and Lindquist, 2000; Wickner, 1994). In addition, yeast prions also exist as different strains, called variants, that can be distinguished on the basis of distinct phenotypic and biochemical

characteristics (Bradley *et al.*, 2002; Bradley and Liebman, 2003; Derkatch *et al.*, 1996; Schlumpberger *et al.*, 2001).

Sup35 is a translational termination factor in its normal soluble form. However, in its prion aggregated [*PSI*$^{+}$] state, its ability to terminate translation at stop codons becomes inefficient. Thus the phenotype of [*PSI*$^{+}$] is suppression of nonsense mutations and in some variants this can cause toxicity and slow growth (Cox *et al.*, 1988; McGlinchey *et al.*, 2011). Sup35 can be divided into three domains. The C-terminal domain is essential for viability and function whereas the N-terminal domain, also called the prion domain, is necessary to form and join prion aggregates and has a non-prion non-essential function in general mRNA turnover (Derkatch *et al.*, 1996; Hosoda *et al.*, 2003; Ter-Avanesyan *et al.*, 1993). The middle domain (M) is required for the faithful maintenance of certain [*PSI*$^{+}$] prion variants (Bradley and Liebman, 2004; Liu *et al.*, 2002).

Overexpression of full length Sup35, or a fragment containing the prion domain of Sup35 (Sup35NM), causes the appearance of [*PSI*$^{+}$] in the presence of [*PIN*$^{+}$] (Derkatch *et al.*, 1996; Derkatch *et al.*, 1997; Derkatch *et al.*, 2001). This efficient [*PSI*$^{+}$] induction phenomenon is explained by a cross-seeding model, which proposes that the preexisting prion, [*PIN*$^{+}$], templates the initial conversion of soluble Sup35 protein molecules to the [*PSI*$^{+}$] prion form (Choe *et al.*, 2009; Derkatch *et al.*, 2001; Derkatch *et al.*, 2004).

Overexpression of Sup35NM fused to GFP (Sup35NM-GFP) in [*psi*$^-$] [*PIN*$^+$] cells causes the appearance of ring or line-like fluorescent aggregates. Cells with such aggregates give rise to [*PSI*$^+$] progeny (Zhou *et al.*, 2001). These newly appearing ring-like aggregates are initially localized at the cell periphery. After about 20-22 hrs the rings shrink to surround the vacuole and sometimes collapse into a dot found at the perivacuolar region. While these large structures never leave the mother cell, daughters appear to inherit [*PSI*$^+$] seed that is too small to be seen using a fluorescence microscope. This seed enables the daughter to propagate [*PSI*$^+$] and directly form a large dot, in the presence of overexpressed Sup35NM-GFP (Fig. 1) (Ganusova *et al.*, 2006; Mathur *et al.*, 2010). Cells that do not form rings were never observed to give rise to [*PSI*$^+$] daughter cells. Also, overexpression of Sup35NM-GFP in already established [*PSI*$^+$] cells gives rise to cells with big fluorescent dots, never rings. Thus ring like aggregates are the hallmark of newly appearing [*PSI*$^+$] following Sup35NM-GFP overexpression in [*PIN*$^+$] cells. Similarly, when Sup35NM-GFP was constitutively overexpressed in the absence of endogenous *SUP35NM*, rings appeared that upon continued propagation converted into large dots. These rings overlapped preautophagosomal markers characteristic of the insoluble protein deposit (IPOD). Rings were composed of long fiber bundles and lysates of the ring cells could transmit [*PSI*$^+$] when the ring fibers were fragmented (Tyedmers *et al.*, 2010).

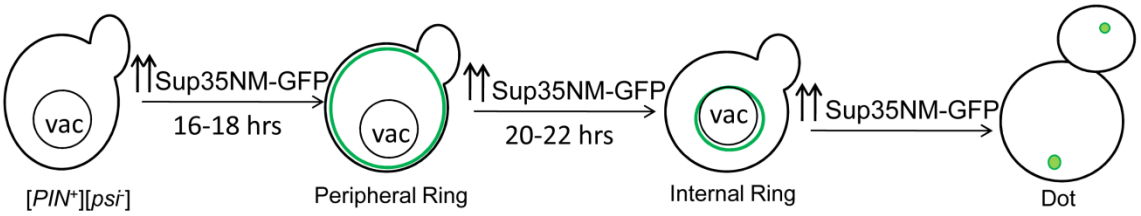

Figure1. Stages during *de novo* induction of [*PSI*$^{+}$]. Previous work showed that when Sup35NM-GFP is over expressed in 74D-694 [*psi*$^{-}$][*PIN*$^{+}$] cells, fluorescent line or ring like structures appear along the cell periphery in about 16-18 hrs. If the induction is prolonged for another 4 hrs, the peripheral ring often becomes an internal ring surrounding the vacuole before collapsing into a dot (Ganusova *et al.*, 2006; Mathur *et al.*, 2010; Zhou *et al.*, 2001).

Interestingly, different [*PSI*$^+$] variants were obtained following overexpression of Sup35NM even in the same genetic background (Derkatch *et al.*, 1996). Variants of [*PSI*$^+$] can be distinguished by differences in levels of Sup35 aggregation and hence differences in translation termination efficiency and toxicity, as well as differences in stability, aggregate structure and oligomer size (Derkatch *et al.*, 1996; King and Diaz-Avalos, 2004; Krishnan and Lindquist, 2005; Kryndushkin *et al.*, 2003; McGlinchey *et al.*, 2011; Tanaka *et al.*, 2004; Toyama *et al.*, 2007; Uptain *et al.*, 2001). In addition variants differ in their responses to alterations in chaperone levels and their ability to be transmitted across transmission barriers (Kushnirov *et al.*, 2000a; Kushnirov *et al.*, 2000b). Strong [*PSI*$^+$] variants have a larger number of aggregates and these aggregates are smaller than the larger, less frequent aggregates found in weak [*PSI*$^+$] variants. Thus, there are more aggregate ends available in strong [*PSI*$^+$] variants to recruit soluble Sup35, resulting in the stronger nonsense suppression phenotype (Derkatch *et al.*, 1996). There are also variants of [*PSI*$^+$] that are intermediate in phenotype between strong and weak [*PSI*$^+$] and all strong or all weak [*PSI*$^+$] are not identical (King, 2001; Kochneva-Pervukhova *et al.*, 2001).

Once established, prion variants generally do not interconvert (Derkatch *et al.*, 1996; Kochneva-Pervukhova *et al.*, 2001). However, weak [*PSI*$^+$] prion variants have been shown to switch to strong [*PSI*$^+$] in the presence of epigallocatechin-3-gallate (EGCG). Whether this small molecule promotes remodeling of the weak

[*PSI*$^{+}$] prion or selects for low levels of EGCG-resistant strong [*PSI*$^{+}$] prions that were present in some of the weak [*PSI*$^{+}$] variant cells is unknown (Roberts *et al.*, 2009). If the latter is true it suggests that prion variants can occasionally spontaneously “mutate” to another variant. When two variants are present in a single cell, and depending upon their relative numbers, the variant that generates seeds more rapidly is believed to cause loss of the other variant, by more efficiently capturing available soluble protein. (Bagriantsev and Liebman, 2004; Bradley *et al.*, 2002; Tanaka *et al.*, 2006).

It remained unknown, if the different [*PSI*$^{+}$] variants induced by transiently overexpressing Sup35NM always arose in separate cells, or if more than one variant could arise in a single cell. One hypothesis was that the ring aggregates in each cell that arose during [*PSI*$^{+}$] induction were composed of a single [*PSI*$^{+}$] variant conformation—resulting from the first seed to appear and grow in that cell, so that different variants could only be initiated in different cells. Here we test this hypothesis and show, to the contrary, that more than one [*PSI*$^{+}$] variant can arise from a single ring cell.

C. Materials and methods

Yeast strains, plasmids and growth conditions

The [*psi*$^-$][*pin*$^-$] and [*PIN*$^+$] strains used in this study are derivatives of 74-D694 (*MAT***a** *ade1-14 leu2-3,112 his3-Δ200 trp1-289 ura3-52*) and are listed in Table 1. Whenever [*PIN*$^+$] was used it was always the "high" variant type (Bradley *et al.*, 2002). *Saccharomyces cerevisiae* strains were grown at 30ºC using standard media and cultivation procedures (Sherman, 1986). Complex media contained 2% dextrose (YPD) or 2% glycerol (YPG). Synthetic media (SD) contained 2% dextrose and appropriate amino acids. The lithium acetate method was used for yeast transformation (Gietz and Woods, 2002).

Plasmid p1182 (*pCUP1-SUP35NM::GFP*) carries the selectable marker *LEU2* and the Sup35NM-GFP fusion under the *CUP1* promoter and is used to induce [*PSI*$^+$] *de novo* (Zhou *et al.*, 2001). Strains transformed with *pCUP1-SUP35NM::GFP* were maintained on synthetic complete medium lacking leucine (–Leu).

Table 1: Yeast strains used in this study

Strains	Description	Source
74D-694	*MAT* **a** *ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200*	(Derkatch *et al.*,1996)
L1749	74D-694 [*psi*$^-$] High [*PIN*$^+$]	(Derkatch *et al.*,1996)
L1758	74D-694 High [*PIN*$^+$] Weak [*PSI*$^+$]	(Bradley *et al*., 2002)
L1762	74D-694 High [*PIN*$^+$] Strong [*PSI*$^+$]	(Bradley *et al*., 2002)
L1998	*MAT***α** *ade2-1 SUQ5 lys1-1 his3-11, 15 leu1 kar1-1 cyhR* High [*PIN*$^+$]	(Bradley *et al*., 2003)
GF658	*MAT***α** *ade1-14 trp1-289 his3-200 ura3-52 leu2-3,112 SUP35-GFP* [*psi*$^-$]	(Satpute-Krishnan *et al*., 2005)
L3102	*MAT***α** *ade1-14 trp1-289 his3-200 ura3-52 leu2-3,112* [*psi*$^-$] *SUP35-GFP,rnq1::HIS3*	(This study)
L2717	*MAT***α** *ade1-14 leu2-3112 ura3-52 trp1-289 lys9-A21* Strong [*PSI*$^+$]	(Vishveshwara *et al*.,2009)

Determination of [*PSI*$^+$] variants arising from single ring containing cells

To induce ring aggregates or [*PSI*$^+$], Sup35NM-GFP [*PIN*$^+$] transformants were grown in plasmid selective (–Leu) media supplemented with 50-150 µM $CuSO_4$ overnight.

To isolate unbudded ring cells, since YPD is auto fluorescent, micromanipulation was done on a thin noble agar pad which was then placed on a YPD plate for further growth. After 2-3 days when the colonies grew to 2-3 mm in diameter, a portion of them was spread on YPD at a concentration of ~200 cells per plate.

To distinguish [*PSI*$^+$] colonies from Mendelian suppressor mutations, individual cells were grown into colonies on 5mM guanidine hydrochloride (GuHCl) and then checked for loss of suppression by spotting on –Ade, YPD and YPG media (Bradley *et al.*, 2003; Tuite *et al.*, 1981). GuHCl eliminates prions by inactivating the chaperone Hsp104, whereas suppressors remain unaffected (Jung and Masison, 2001).

[*PSI*$^+$] color assay

All yeast strains used have the *ade1-14* allele that has a nonsense mutation and is frequently used to score for [*PSI*$^+$] (Chernoff *et al.*, 1995). Normal [*psi*$^-$] cells with the functional Sup35 translation termination factor terminate protein translation

efficiently at the premature *ade1-14* nonsense codon, which causes cells to be Ade$^-$ and to accumulate red pigment on rich medium like YPD. In contrast, in [*PSI*$^+$] cells, aggregation and thus inactivation of Sup35 causes some read through of the *ade1-14* premature stop codon, so some full length Ade1 is synthesized giving Ade$^+$ white (strong [*PSI*$^+$]), pink (weak [*PSI*$^+$]) or sectored (unspecified [*PSI*$^+$]) colonies.

We examined the effects of all plasmids used in this study on the color of [*PSI*$^+$] cells and found no effects. This was important because a Gal-Sup35 plasmid we used in a previous study caused anti-suppression even when the Gal promoter was turned off on glucose. The presence of this plasmid caused strong [*PSI*$^+$] cells to grow into pink colonies on YPD, that had white sectors whenever the plasmid was lost (Patel and Liebman, 2011).

Fluorescent microscopy and quantification of cytoplasmic Sup35NM-GFP levels

Fluorescent images were acquired with a Zeiss Axioskop 2 microscope and an AxioCam digital camera (Carl Zeiss), and processed with AxioVision software (Carl Zeiss). For quantification, L1749 transformed with *pCUP1-SUP35NM::GFP* and grown overnight in –Leu medium containing 50 µm $CuSO_4$ was washed and grown in YPD for another 3 hrs. Images were acquired from randomly chosen ring containing cells with a single attached bud and from control [*psi*$^-$] cells with Sup35 endogenously tagged with GFP (kindly supplied by T. Serio) (Satpute-Krishnan and Serio, 2005). The fluorescence intensity was determined with image J software

(Rasband, 1997-2012). An interior region of the cell excluding the vacuole was selected with the “brush” tool. The mean background intensity of an area next to each cell was subtracted from the cell’s mean fluorescence intensity to get the actual value for that cell.

Pedigree analysis

Micromanipulation of individual ring containing cells was done on a thin 2% noble agar pad which was transferred to a YPD plate where it was allowed to divide for 6-10 hrs. The agar pad was then removed from the plate and the cells were examined under the fluorescent microscope and were separated on the agar pad. The pad was then returned to a YPD plate and the separated cells were allowed to form colonies which were then respread on YPD to score for [*PSI*$^{+}$] variants on the basis of colony color.

Biochemical analysis of yeast lysates

Cell lysates were prepared from 50 ml of overnight culture, by vortexing cells in 750 µl of lysis buffer (80 mM Tris, 300 mM KCl, 10 mM $MgCl_2$ and 20 % [wt/vol] glycerol, 1:50 diluted protease inhibitor cocktail [Sigma], and 5 mM PMSF) at pH 7.6 with 0.5 mm glass beads (Biospec) at high speed five times for 1 min separated with 1 min cooling in ice. Lysates were precleared of cell debris by centrifuging two times at 600 g for 1 min at 4ºC (Kushnirov *et al.*, 2006).

To analyze [*PSI*$^{+}$] aggregates by SDD-AGE, ~40 µg of crude lysate was treated with 2 % SDS sample buffer (25 mM Tris, 200 mM glycine, 5 % glycerol, and 0.025 % bromophenol blue) for 7 min at room temperature, electrophoretically resolved in a horizontal 1.5 % agarose gel in a standard tris/glycine/SDS buffer, transferred to a polyvinylidene difluoride membrane and probed with Sup35C antibody as described previously (Bagriantsev *et al.*, 2006).

Cytoduction

Cytoduction was carried out between [*RHO*$^{+}$] donors and mitochondrial deficient [*rho*$^{-}$] recipients. Either donor or recipient carried a *kar1* mutation that inhibits nuclear fusion (Conde and Fink, 1976). Following mating, cytoductants and diploids were selected by growth on synthetic media lacking amino acids specifically required by the donor and using glycerol as the sole carbon source where functional mitochondria are required for growth. Thus the cytoductant would inherit the nucleus from one parent and mitochondria from another. Cytoductants were distinguished from diploids on the basis of their auxotrophic markers.

Propagon analysis

The qualitative and quantitative analysis of propagons per cell was done by using the previously described *in vivo* propagon dilution method (Cox *et al.*, 2003).

[*PSI*$^+$] variants study in zygotes

Strong [*PSI*$^+$] (L2717) (Vishveshwara *et al.*, 2009) and weak [*PSI*$^+$] (L1758) were mated overnight on YPD plates and individual micromanipulated zygotes were grown overnight on YPD. The resulting microcolonies which were then suspended in water and spread on YPD where pink and white colonies were respectively scored as weak vs. strong [*PSI*$^+$]. Colonies were confirmed to be diploid by marker analysis.

D. Results:

1. [*PSI*$^+$] variants are not always established at the ring stage

To investigate when different variants of [*PSI*$^+$] are established during the induction of [*PSI*$^+$], we transiently overexpressed Sup35NM fused with GFP (Sup35NM-GFP) in 74D-694 [*PIN*$^+$] [*psi*$^-$] cells carrying the *pCUP1-SUP35NM::GFP* (*LEU2*) plasmid. As shown previously, this transient overexpression gave rise to cells containing bright fluorescent rings indicative of the ability to give rise to [*PSI*$^+$] (Fig. 2) (Zhou *et al.*, 2001).

Individual ring containing cells were isolated by micromanipulation and grown into colonies on rich, glucose containing medium (YPD) for 3 days. To determine the [*PSI*$^+$] variant status of the cells in these colonies, they were suspended in water and individual cells were again grown into colonies on YPD. The color of these colonies reflected the [*PSI*$^+$] variant status of the suspended cells because the

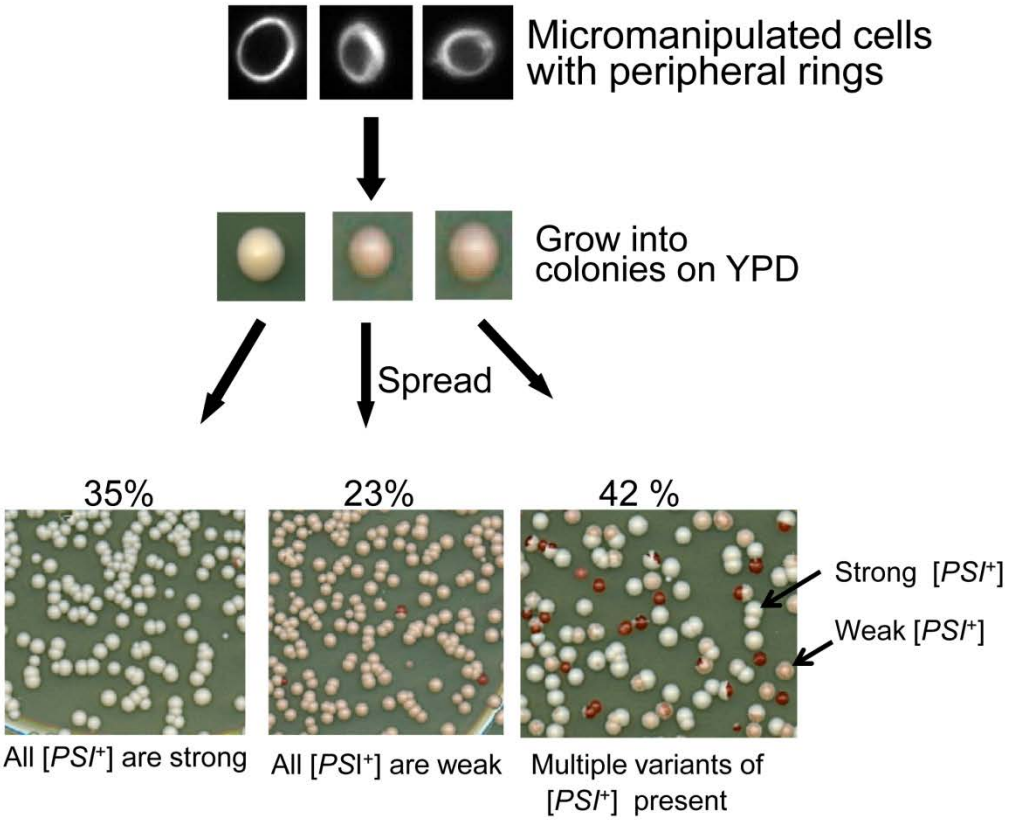

Figure 2. More than one [*PSI*$^+$] variant can arise from a single cell following *de novo* induction of [*PSI*$^+$]. One ring cell can give rise to progeny that are all strong [*PSI*$^+$], all weak [*PSI*$^+$] or a mixture of strong and weak [*PSI*$^+$]. [*PSI*$^+$] was induced *de novo* by over expressing Sup35NM-GFP in [*PIN*$^+$] [*psi*$^-$] cells. Cells with rings were micromanipulated and grown on YPD plates for 3 days where Sup35NM-GFP expression was turned off. The resulting colonies were suspended in water and spread on YPD. The types of [*PSI*$^+$] variants present in the ring cell progeny were determined from the color of these colonies (>500 ring containing cells were micromanipulated). Only ring cells giving rise to some [*PSI*$^+$] progeny are depicted.

ability to read through the premature stop codon in the *ade1-14* mutation caused [*PSI*$^{+}$] cells to turn from a dark red color to white, in proportion to the level of read through (see Materials and Methods). These viable ring containing cells frequently (~60%) gave rise to some [*PSI*$^{+}$] progeny, although ~40% of the ring cells had only [*psi*$^{-}$] progeny. Most (95%) of the non-red [*PSI*$^{+}$] colonies among the progeny failed to grow when transferred to –Leu, indicating that the plasmid was lost during growth on the non-selective YPD medium. Since it is difficult to distinguish all [*PSI*$^{+}$] variant types, we focused on phenotypically distinct strong (white) and weak (pink) [*PSI*$^{+}$] variants. We found that of the ring cells isolated after 17 hrs of induction that gave rise to some [*PSI*$^{+}$] progeny, ~60 % of the time the [*PSI*$^{+}$] progeny were either all strong or all weak [*PSI*$^{+}$], but ~40% of the time the [*PSI*$^{+}$] progeny clearly included a mixture of variants including both strong and weak [*PSI*$^{+}$] (Fig. 2). The proportion of the two variants in the mixture fell between the range of 50:50 and 90:10.

The *de novo* induction of [*PSI*$^{+}$] is caused by high levels of Sup35NM-GFP and only the ring containing cells, not their daughters have these high levels. Indeed, while the level of Sup35NM-GFP in ring cells is 10X higher than the level of endogenous Sup35, when ring cells are placed on non-inducing media their daughters contain a much lower level of Sup35NM-GFP, less than half of the endogenous Sup35 level (Fig. 3). This is not surprising because most of the Sup35NM-GFP fluorescence is confined to the ring and is not diffuse within the cytoplasm and therefore not easily transmissible to daughter cells. Sup35NM-GFP

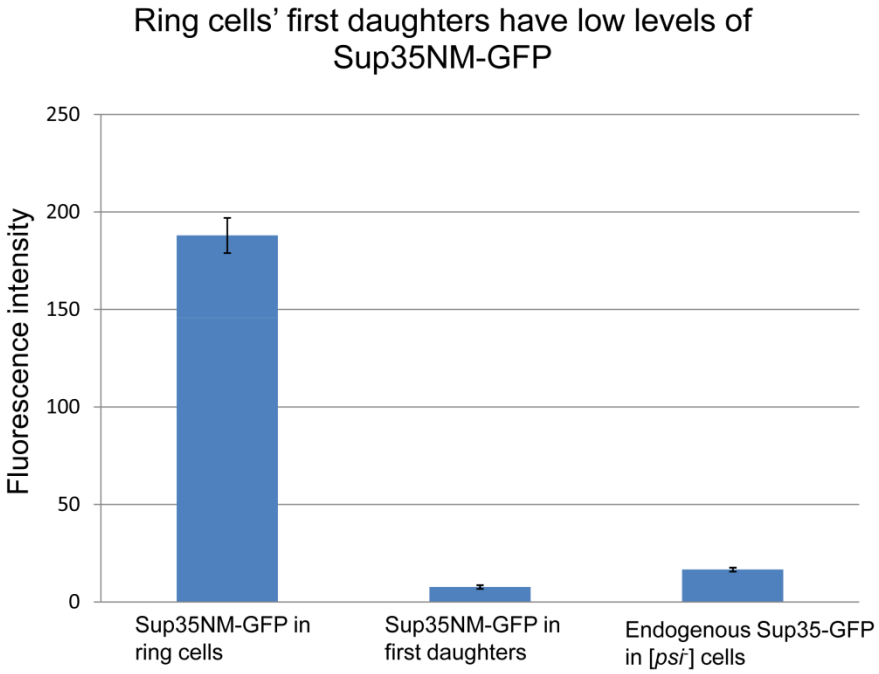

Figure 3. Ring cells' first daughters have low levels of Sup35NM-GFP. Comparison of endogenous Sup35-GFP fluorescence in [*psi*⁻] cells with cytoplasmic Sup35NM-GFP fluorescence in ring containing mother cells and their first daughters. Images of GF-658 (*MAT* **α** *ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200 SUP35-GFP*) were taken for the [*psi*⁻] cells. Ring cells and daughters of ring cells were imaged from L1749 (*MAT* **a** *ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200*) transformed with *pCup-SUP35NM-GFP* after induction until ring stage. After induction and the appearance of rings, cells were washed with water and grown in YPD for ~ 3 hrs. Images were then acquired and quantified from three different trials of 10 cells each (see Experimental Procedures). All cells were imaged under identical conditions Bar graphs represent the average fluorescence intensity of Sup35-GFP and Sup35NM-GFP in the respective cells. The level of Sup35-GFP was reduced about 25% relative to the level of untagged Sup35 in Western blots (data not shown). This may reflect differential degradation in the lysate.

levels were estimated by comparing the fluorescence intensities of Sup35NM-GFP in ring containing mothers and their daughters with control [*psi*$^-$] cells containing endogenous Sup35 tagged with GFP (Satpute-Krishnan and Serio, 2005). Thus, it appears that a heterogeneous mixture of [*PSI*$^+$] aggregates, capable of giving rise to different variants of [*PSI*$^+$] can be initially formed in a single cell following Sup35NM-GFP overexpression. Indeed, the mixture of variants does not result from an induced change of one variant type into another because overexpressing of Sup35NM-GFP in the presence of either strong or weak [*PSI*$^+$] did not cause a phenotypically distinguishable new variant to appear (Fig. 4).

[*PSI*$^+$] aggregates are composed of oligomeric species which are comparatively more stable than normal protein aggregates. These oligomers are SDS resistant at room temperature and their sizes can be estimated on agarose gels by semi-denaturing gel electrophoresis (SDD-AGE) (Bagriantsev and Liebman, 2004; Kryndushkin *et al.*, 2003). We show that [*PSI*$^+$] cells initially derived from a single ring cell and that all appear alike on plates had a similar oligomer size distribution (Fig. 5 left, center). Also as expected, the pink and white colonies derived from a single ring cell displayed the different oligomer sizes characteristic for weak and strong [*PSI*$^+$] respectively (Fig. 5 right).

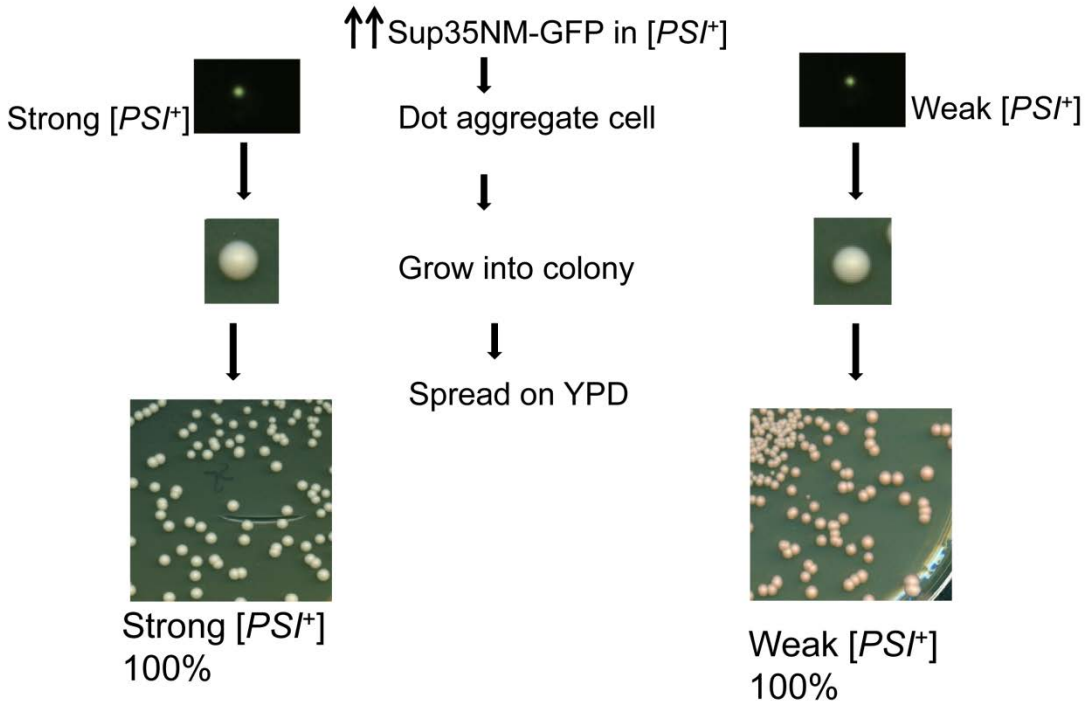

Figure 4. **Overexpression of Sup35NM-GFP does not affect already established [*PSI*$^+$] variants**. As a control experiment Sup35NM-GFP was over expressed in strong [*PSI*$^+$] and weak [*PSI*$^+$] cells. Single cells containing bright fluorescent dots were micromanipulated and allowed to form colonies that were spread on YPD for the color assay (200 strong [*PSI*$^+$] and 200 weak [*PSI*$^+$] cells were micromanipulated).

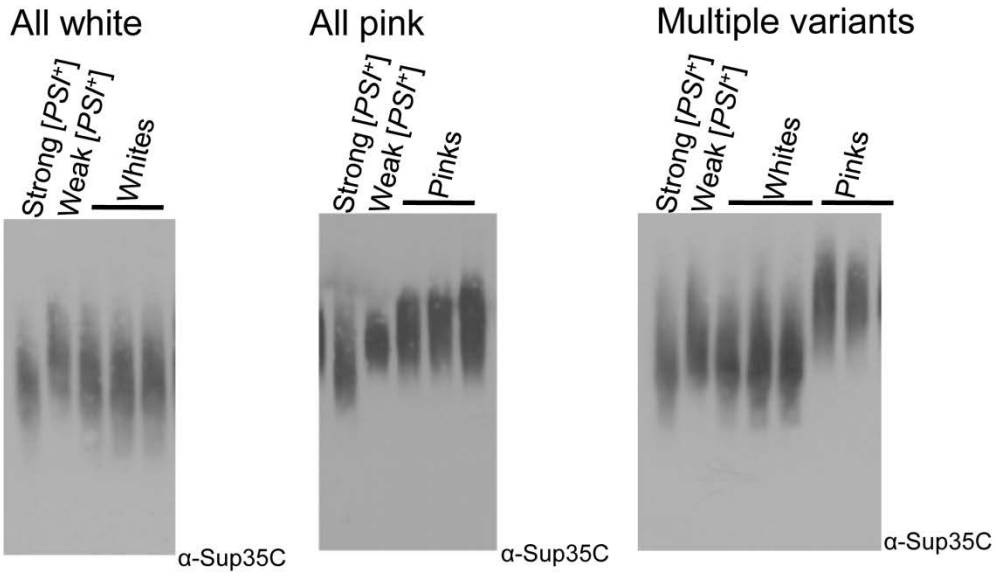

Figure 5. Confirmation that different color colonies have variants with different sized Sup35 oligomers. Cell lysates were prepared from colonies shown at the bottom of 2A. Crude lysates were treated with 2% SDS at room temperature and SDS resistant oligomers were analyzed by SDD-AGE analysis. Lysates from strong [*PSI*$^{+}$] (L1762) and weak [*PSI*$^{+}$] (L1758) variants were run as controls. Left panel shows results from ring cells whose [*PSI*$^{+}$] progeny were all white, center shows results from ring cells whose [*PSI*$^{+}$] progeny were all pink and right panel shows results from ring cells whose [*PSI*$^{+}$] progeny were both white and pink.

2. [*PSI*$^+$] establishment can be altered by increasing the duration of Sup35NM-GFP overexpression

To investigate whether the duration of induction of Sup35 is crucial in determining the resulting [*PSI*$^+$] variant, we compared the [*PSI*$^+$] variant status of progeny of individual ring containing cells micromanipulated after 17 vs. 24 hrs of Sup35NM-GFP induction. As shown above, after 17 hrs of induction, ~ 40% of the [*PSI*$^+$] generating ring cells (corresponding to 25% of total cells) gave rise to both strong and weak [*PSI*$^+$] progeny. This fraction was reduced to 20% (12.5% of total) when ring cells were micromanipulated after 24 hrs of induction (Fig. 6). At both times ~ 60 % of the viable ring cells gave rise to some non-red [*PSI*$^+$] colonies. Likewise at both times, of the cells giving rise to a single [*PSI*$^+$] phenotype, ~60% were strong [*PSI*$^+$] and ~40% were weak [*PSI*$^+$]. Thus, with longer Sup35NM-GFP overexpression, fewer cells retain the ability to give rise to both strong and weak [*PSI*$^+$] but the level of [*psi*$^-$] cells is unchanged. In contrast, altering the overexpression levels of Sup35NM by increasing the concentration of copper had no effect in the pattern of [*PSI*$^+$] variant establishment at either time point (Fig. 7).

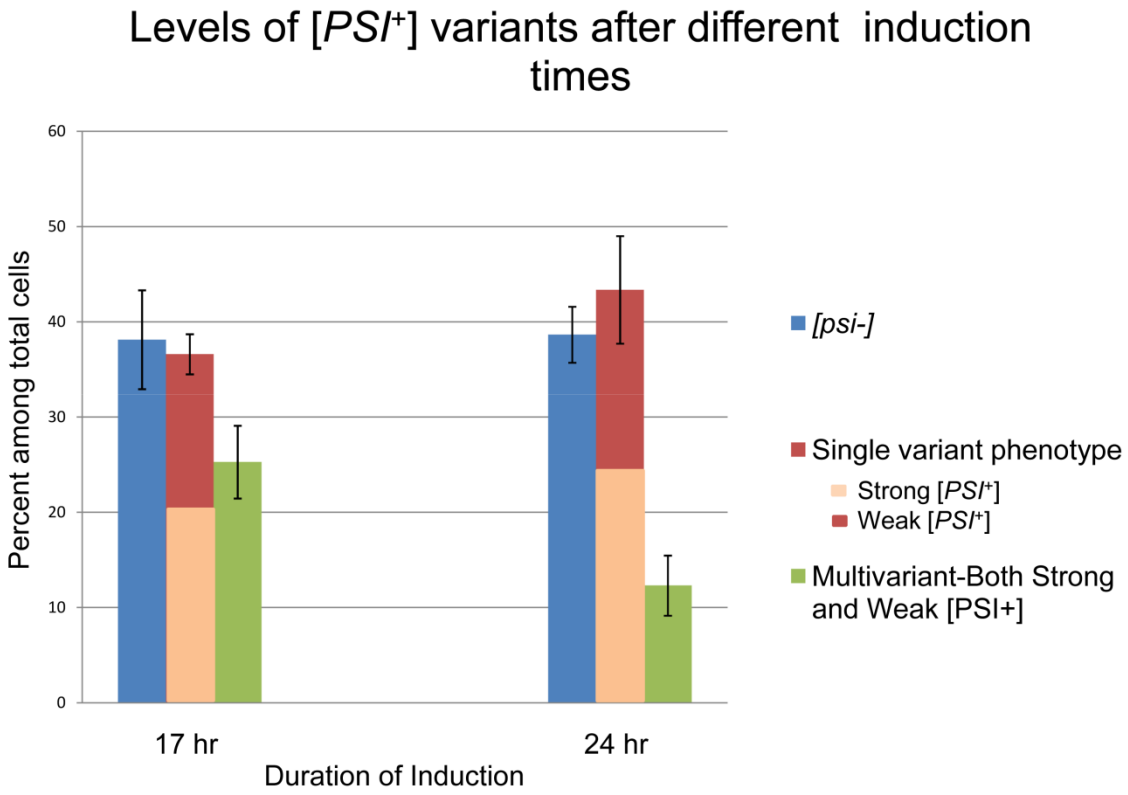

Figure 6. [*PSI*$^{+}$] variant establishment varies with the duration of Sup35NM protein induction. [*PIN*$^{+}$] [*psi*$^{-}$] cells containing the Sup35NM-GFP plasmid were grown in 50 µM $CuSO_4$ at 30ºC for 17 or 24 hrs to induce [*PSI*$^{+}$]. Ring aggregate containing cells were then isolated and progeny examined. Ring cells gave rise to [*PSI*$^{+}$] variants with a single phenotype more frequently after 24 vs. 17 hrs of Sup35NM-GFP expression. Each bar represents standard error of more than 3 trials of at least 100 viable ring cells. Data includes all ring cells whether or not they gave rise to any [*PSI*$^{+}$].

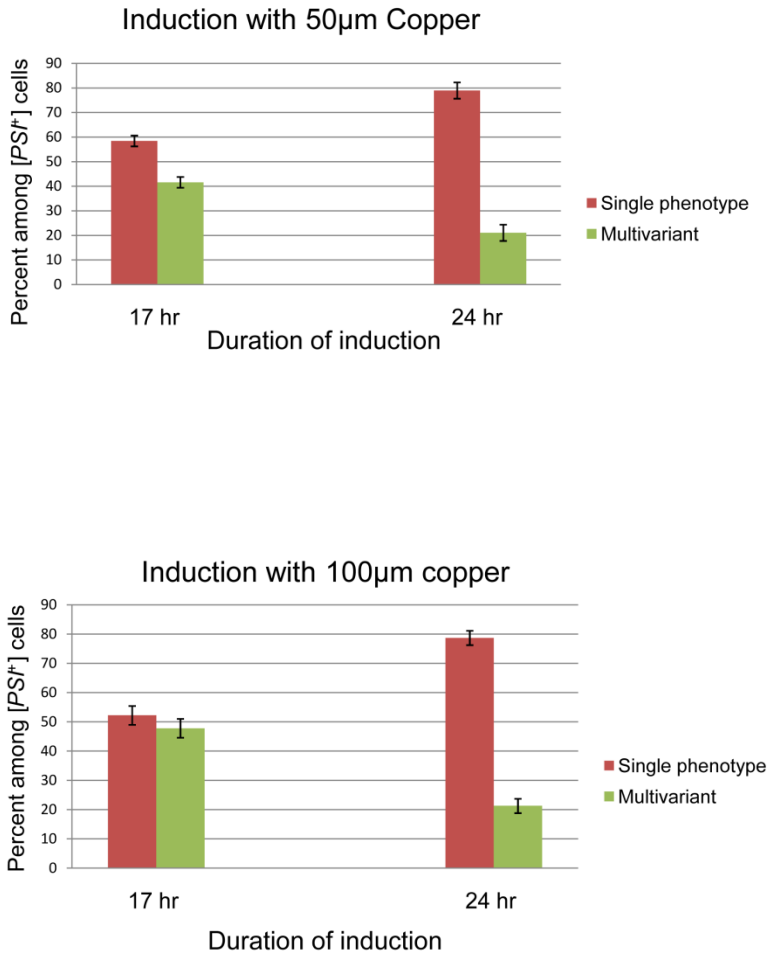

Figure 7. Increasing expression of Sup35NM-GFP does not alter the variant establishment. Individual ring cells were micromanipulated after 17 or 24 hrs of induction with 50 µM or 100 µM of CuSO4 and the [*PSI*$^{+}$] variant types arising from these cells were determined. No significant difference was observed in the relative frequencies of [*PSI*$^{+}$] variants whether 50 or 100 µM CuSO4 was used. Each bar indicates standard error of more than three trials of 100 viable ring cells. Data shown includes only ring cells giving rise to some [*PSI*$^{+}$] progeny. (For all experiments starting OD at 0 hrs=0.1, OD at 17 hrs~1.0 and OD at 24 hrs ~2.0 unless otherwise indicated).

3. Weak or strong [*PSI*$^+$] variants usually get established within the first few divisions of ring cells

Our findings show that single [*PSI*$^+$] phenotypes do not always get established with the initial formation of ring aggregates. Thus to determine when a single phenotype gets established, we analyzed pedigrees of ring containing cells. Individual ring cells were micromanipulated after 17 hrs of Sup35NM-GFP overexpression and allowed to divide a few times. These cells were then separated and grown into colonies on YPD where their [*PSI*$^+$] status was determined by color. We examined 150 unbudded single ring cells: 90 cells did not divide, 19 had all [*psi*$^-$] daughters, 31 had all strong or weak [*PSI*$^+$] daughters, and 10 had both weak [*PSI*$^+$] and strong [*PSI*$^+$] daughters. In 8 of the pedigrees of the latter 10 ring cells, a daughter, granddaughter or great granddaughter lost the ability to transmit either strong or weak [*PSI*$^+$] to their daughters. In the example in Figure 8, a weak [*PSI*$^+$] variant became established in a granddaughter. In this pedigree the mother and one daughter cell failed to grow into a colony. The progeny of the other daughter included strong and weak [*PSI*$^+$] but the progeny of a granddaughter were all weak [*PSI*$^+$]. SDD-AGE analysis of the daughter's white and pink progeny showed the expected difference in the size of oligomers. Thus, in this case the variant was established in a granddaughter. In another example (Fig. 9), the ring cell's progeny include cells with strong and weak [*PSI*$^+$] variants. In contrast, the daughter had only strong [*PSI*$^+$] progeny. Thus, the variant was established in the daughter.

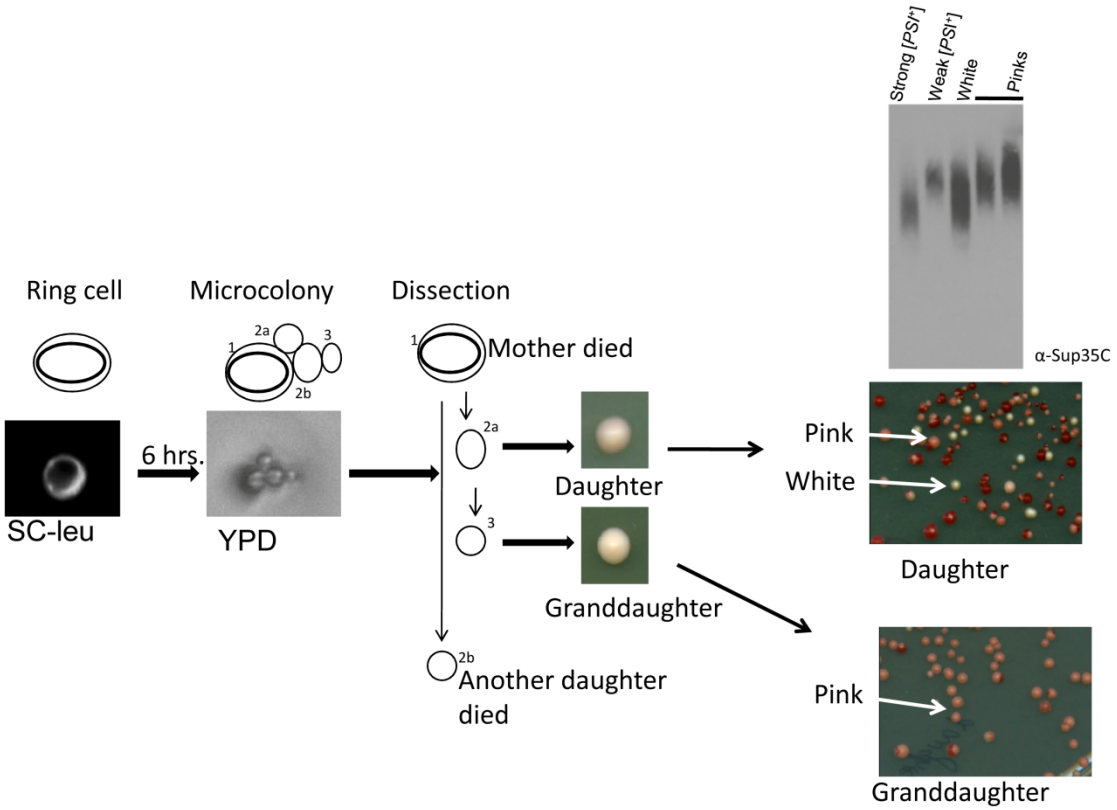

Figure 8. Pedigree analysis to determine when [*PSI*$^+$] variants are established. Cells with *pCup-SUP35NM-GFP* induced to form rings in plasmid selection medium plus Cu^{2+} were micromanipulated on an agar pad, placed on YPD and allowed to divide for a few generations. The resulting cells were dissected (see Materials and Methods) while keeping track of the different generations. The cells were then grown into colonies on YPD which were then spread on YPD to score for the [*PSI*$^+$] variant in the progeny of each cell in the microcolony. **A**. Pedigree in which a weak [*PSI*$^+$] variant was established in a granddaughter. The ring containing mother cell (1) and one daughter cell (2b) did not grow. The other daughter (2a) gave rise to more than one variant. The granddaughter (3) gave rise to only weak [*PSI*$^+$] progeny. The Sup35 SDS resistant oligomer sizes present in the pink and white colonies from daughter (2a) were compared with standard strong [*PSI*$^+$] (L1762) and standard weak [*PSI*$^+$] (L1758). Lysates treated with 2% SDS were subjected to western blotting and probing with Sup35C antibody.

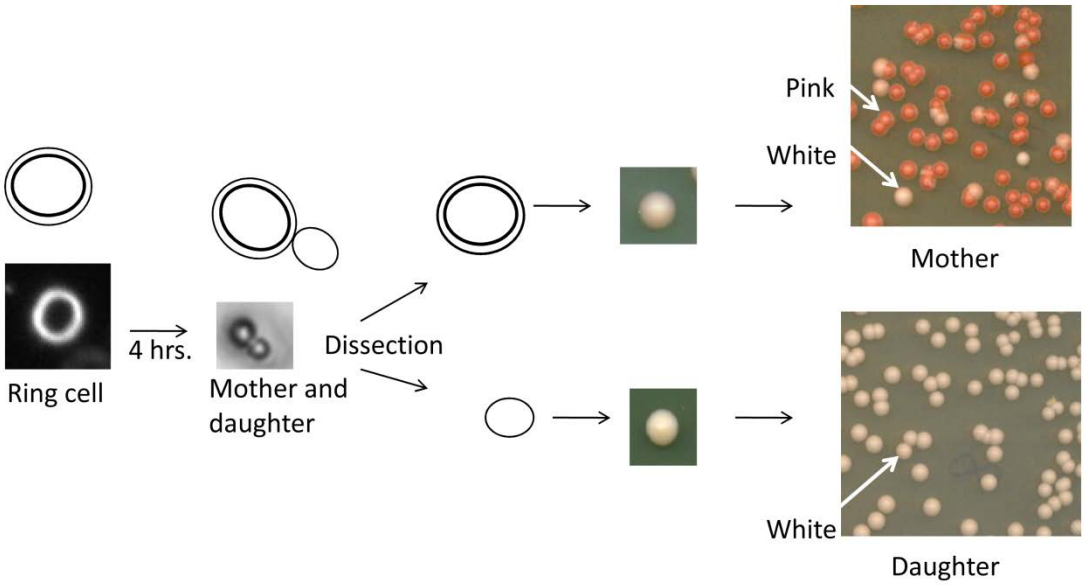

Figure 9. A pedigree in which strong [*PSI*$^{+}$] is established in a daughter. Both weak and strong [*PSI*$^{+}$] were detected in the progeny of the initial aggregate containing mother cell. The daughter only gave rise to strong [*PSI*$^{+}$] progeny.

4. Unspecified [*PSI*$^{+}$]

In contrast to the above 8 pedigrees, in 2 pedigrees all [*PSI*$^{+}$] daughters, granddaughters and great granddaughters transmitted both strong and weak [*PSI*$^{+}$] to some of their progeny. In one such pedigree, a ring cell formed a 14 celled microcolony. When these individual cells were separated and grown into colonies, 2 were [*psi*$^{-}$], while most of the colonies from the other 12 appeared to be dark pink with numerous white sectors, although there were also solid red [*psi*$^{-}$], some solid white (strong [*PSI*$^{+}$]) and occasional solid pink (weak [*PSI*$^{+}$]) colonies (Fig. 10). When the sectored colonies were streaked out, they continued to give rise to mostly pink colonies sectoring white like themselves, as well as some solid red, white and rare pink colonies. Simple observation of the sectoring colonies cannot distinguish if the sectors are pink, white and red or just white and red. However, the fact that subculturing of these colonies did not give large numbers of red colonies suggests that these sectoring colonies are largely pink and white with limited red sectors. We call these sectored colonies 'unspecified [*PSI*$^{+}$]' and refer to strong or weak [*PSI*$^{+}$] as specified [*PSI*$^{+}$] variants. Furthermore when cells picked from white sectors were subcultured they gave rise to non-sectoring white colonies, whereas cells picked from pink sectors grew into a mixture of cells with unspecified, some strong, few [*psi*$^{-}$] and rarely weak [*PSI*$^{+}$]. All of the unspecified [*PSI*$^{+}$] colonies had lost the plasmid, so leaky expression of SUP35-GFP from the plasmid cannot be

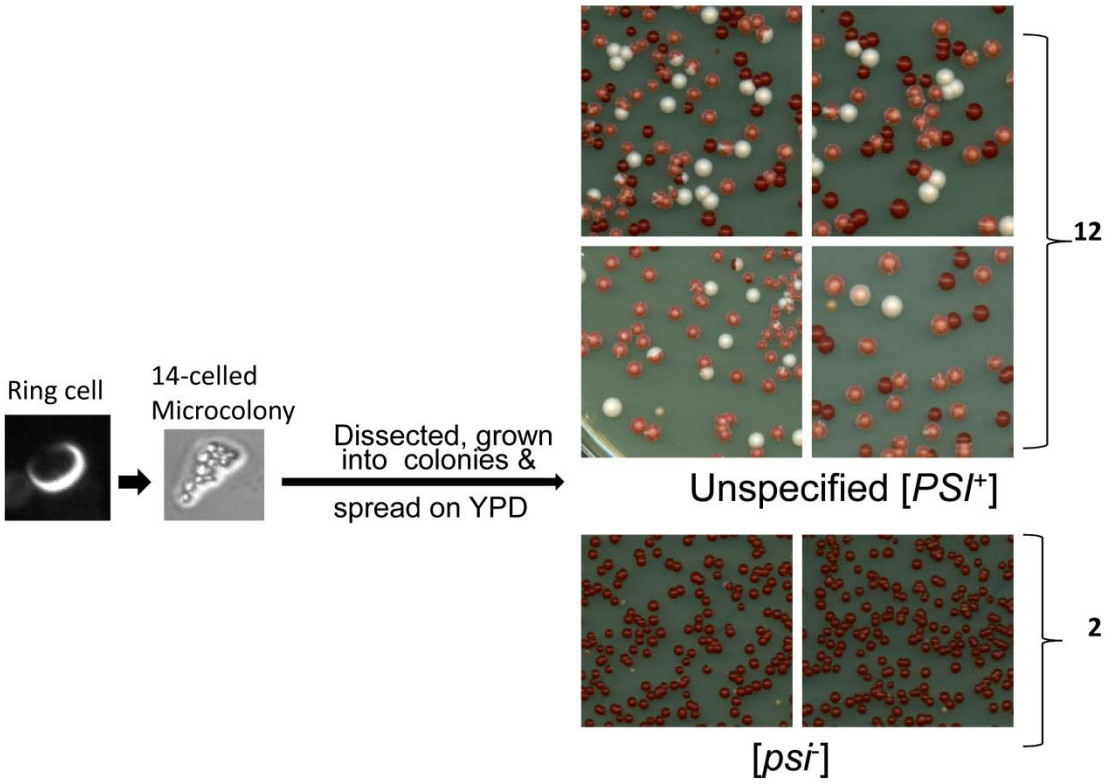

Figure 10. Some [*PSI*$^{+}$] variants remain unspecified. Pedigree of an unspecified [*PSI*$^{+}$] cell. A ring containing cell was grown into a 14 cell microcolony. Each of the cells in the microcolony were separated, grown into colonies, and spread on YPD. Most of the cells from 12 of the colonies grew into dark pink colonies that had white sectors. There were also some [*psi*$^{-}$] red, some solid white (strong [*PSI*$^{+}$]) and occasional solid pink (weak [*PSI*$^{+}$]) colonies seen. The sectoring colonies are called unspecified [*PSI*$^{+}$]. The two other colonies gave rise to only red, [*psi*$^{-}$] cells.

responsible for the unspecified phenotype. Even after 25 sequential subculturings, unspecified [*PSI*$^{+}$] continued to give rise to progeny with more than one variant. Interestingly, with each subculturing there were fewer [*psi*$^{-}$] colonies. The reduced number of [*psi*$^{-}$] colonies correlated with the sectoring colonies becoming lighter and lighter. By the 25th pass the population contained only pink/white sectored (unspecified [*PSI*$^{+}$]), white (strong [*PSI*$^{+}$]) and pink (weak [*PSI*$^{+}$]) colonies but no red ([*psi*$^{-}$]) cells (Fig. 11).

This type of unspecified [*PSI*$^{+}$] was ~ 5 % of the total [*PSI*$^{+}$] induced *de novo.* When Sup35NM-GFP was overexpressed overnight in L1749 and plated on YPD, of about 1,250 non-red colonies scored, 60 had the phenotype of unspecified [*PSI*$^{+}$]; dark pink with numerous white sectors. They were subcultured on YPD for 2 passes, and 52 of them behaved like the unspecified [*PSI*$^{+}$] described above and none contained the plasmid.

5. <u>Unspecified [*PSI*$^{+}$] vs. strong and weak [*PSI*$^{+}$] variants</u>

Like other [*PSI*$^{+}$] variants, unspecified [*PSI*$^{+}$] was cytoducible and was cured by growth in 5 mM GuHCl. Since unspecified [*PSI*$^{+}$] sectored colonies contained strong [*PSI*$^{+}$] and weak [*PSI*$^{+}$] cells, unspecified, strong and weak [*PSI*$^{+}$] cytoductants were expected from the cytoduction mixture. Among 50 [*PSI*$^{+}$] cytoductants scored from a mating of unspecified [*PSI*$^{+}$] donors with the

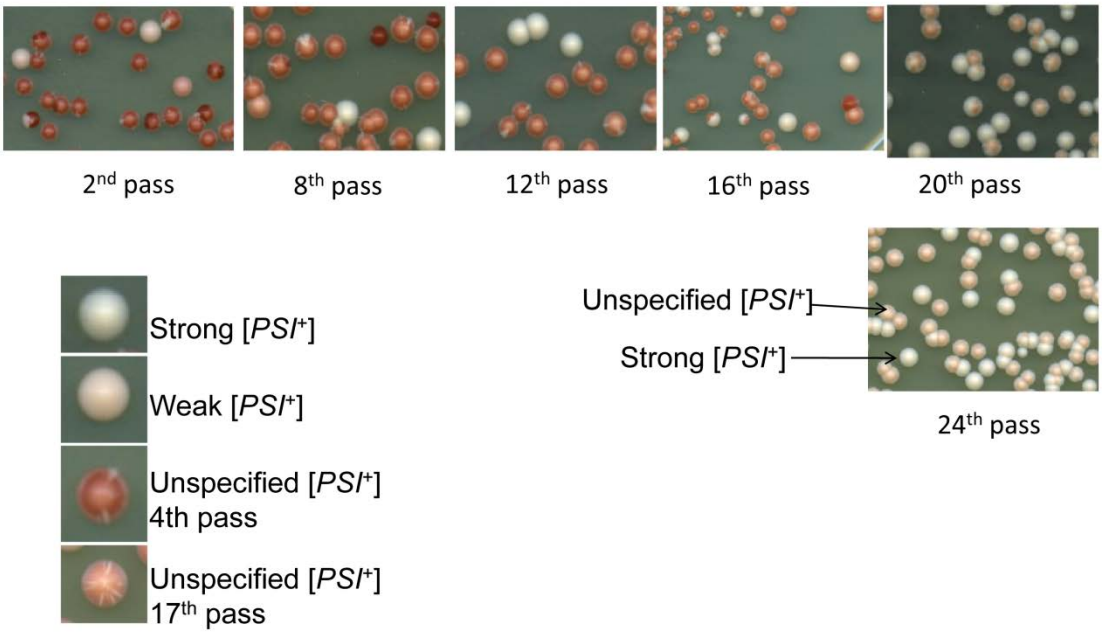

Figure 11. After continued propagation, unspecified [*PSI*$^{+}$] cells have a reduced number of [*psi*$^{-}$] progeny. On subsequent subculturing, unspecified [*PSI*$^{+}$] gave rise to fewer and fewer [*psi*$^{-}$] daughters, but never lost the ability to give rise to unspecified strong and weak [*PSI*$^{+}$] daughters. When it reached the 24th pass it did not give rise to any [*psi*$^{-}$] daughters. Also, the unspecified colony color became lighter with each subculturing but still showed white and pink sectors indefinitely.

[*psi*$^-$][*PIN*$^+$] *kar1* recipient (L1998), 5 were unspecified, 10 were weak and 35 were strong [*PSI*$^+$]. Thus the unspecified [*PSI*$^+$] can be cytoduced and is also maintained in a different genetic background.

Sup35 oligomers from an unspecified [*PSI*$^+$] culture correspond to the oligomer size characteristic of weak [*PSI*$^+$] rather than strong [*PSI*$^+$] (Fig. 12). Overexpression of the Hsp104 chaperone cures [*PSI*$^+$] by an unknown mechanism (Chernoff *et al.*, 1995). We used galactose inducible Hsp104 to increase the level of Hsp104 in unspecified, strong (L1762) and weak (L1758) [*PSI*$^+$] colonies for 1-24 hrs. Curing was measured by plating the samples on YPD to look for the percentage of red [*psi*$^-$] colonies (data not shown). As reported previously, curing by excess Hsp104 is more rapid in weak than strong [*PSI*$^+$] (Derkatch *et al.*, 1996). The time needed for excess Hsp104 to cure unspecified [*PSI*$^+$] was intermediate.

To compare the microscopic appearance of [*PSI*$^+$] aggregates in unspecified, strong (L1762), and weak [*PSI*$^+$] (L1758) strains, we expressed Sup35NM-GFP protein at a low level to stain the aggregates. Either 1-3 big aggregates per cell or numerous tiny aggregates per cell were observed in all three variants.

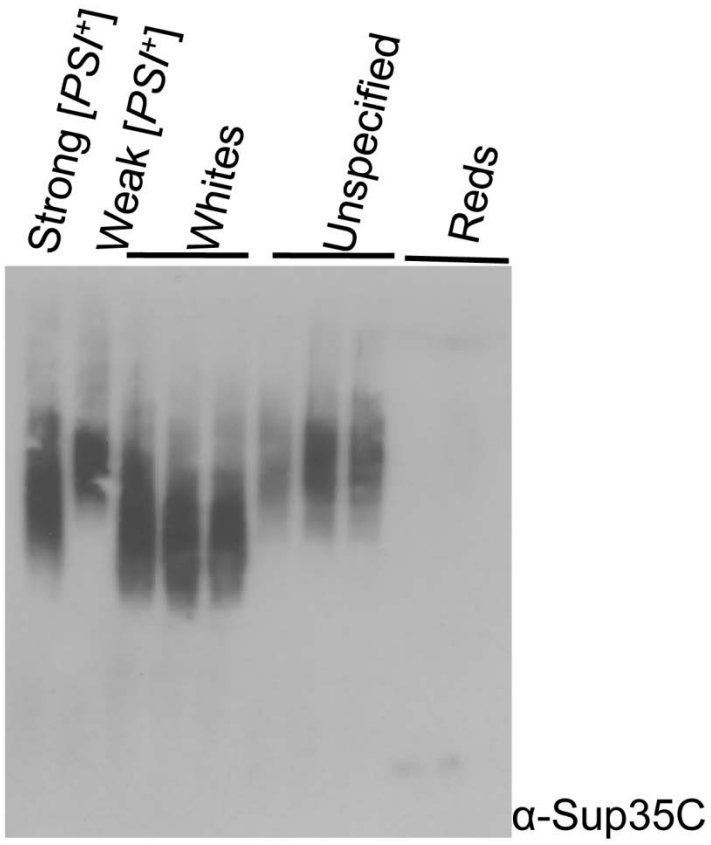

Figure 12. Comparative SDD-AGE analysis of the oligomer size of unspecified [*PSI*$^{+}$].

6. The unspecified [*PSI*$^+$] property is independent of [*PIN*$^+$]

Unspecified [*PSI*$^+$] cells were shown to retain [*PIN*$^+$] because Rnq1 was found in the pellet fractions of lysate when subjected to high speed centrifugation (Fig. 13). To determine if the unspecified [*PSI*$^+$] character is dependent on the presence of [*PIN*$^+$], we asked if unspecified [*PSI*$^+$] could propagate if [*PIN*$^+$] were lost. We crossed unspecified [*PSI*$^+$] with a [*psi*$^-$] *rnq1Δ* strain and examined the [*PSI*$^+$] status of meiotic progeny (Fig. 14). The unspecified character segregated in a non-Mendelian fashion and was found in 7 [*PIN*$^+$] and 8 *rnq1Δ* (which are necessarily [*pin*$^-$]) segregates from 14 tetrads with 4 viable spores and 3 tetrads with 3 viable spores, establishing that the unspecified phenotype is independent of the [*PIN*$^+$] prion.

7. Unspecified [*PSI*$^+$] does not mimic a mixture of weak and strong [*PSI*$^+$] propagons.

Our finding that unspecified [*PSI*$^+$] cells give rise to both specified strong and weak [*PSI*$^+$] progeny could be explained if unspecified [*PSI*$^+$] cells contained a mixture of strong and weak [*PSI*$^+$] propagons. An alternative explanation is that the [*PSI*$^+$] propagons in these cells were truly unspecified and could mature into specified strong or weak [*PSI*$^+$] variants in daughter cells.

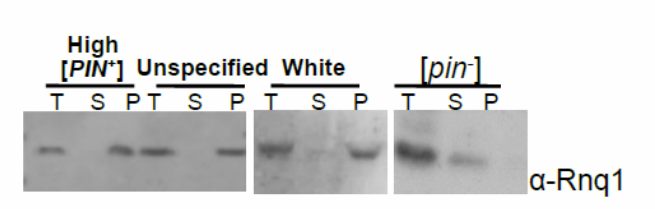

Figure 13. **[*PIN*$^+$] is present in cells with unspecified [*PSI*$^+$].** Lysates from unspecified [*PSI*$^+$] cells, from white daughters of unspecified [*PSI*$^+$] cells and from high [*PIN*$^+$] control cells were subjected high speed centrifugation at 100,000g. Total (T), supernatant (S) and pellet (P) fractions were collected and analyzed by SDS-PAGE and western blotting. Rnq1 is found in the pellet fraction in high [*PIN*$^+$], whereas it is present in the soluble fraction in [*pin*$^-$]. In unspecified [*PSI*$^+$], which contains high [*PIN*$^+$], Rnq1 is present in the pellet fraction.

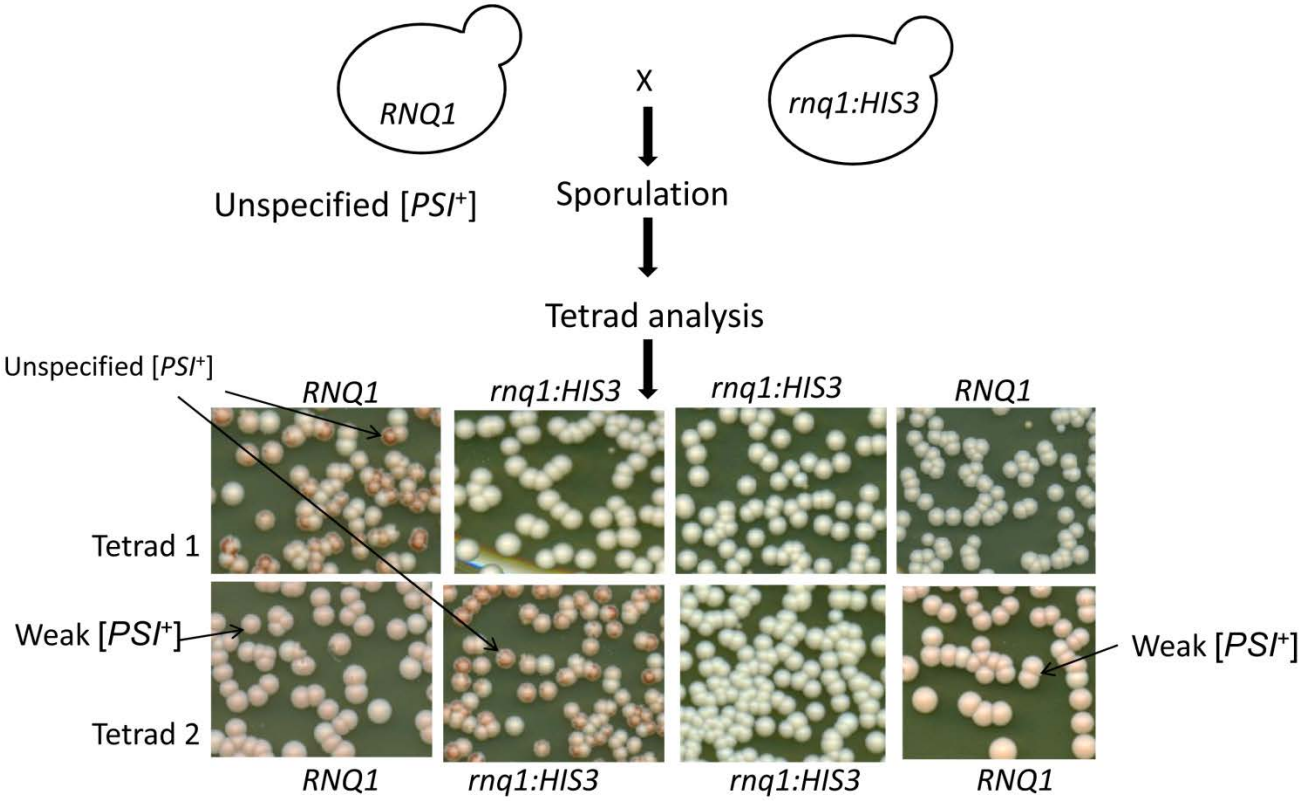

Figure 14. The unspecified [*PSI*$^+$] phenotype is not dependent on [*PIN*$^+$]. Unspecified [*PSI*$^+$] was crossed with an *RNQ1* deletion strain L3102 ([*psi*$^-$] *SUP35-GFP, rnq1::HIS3*) and diploids were sporulated. The progeny from two of the 17 tetrads examined are shown where unspecified [*PSI*$^+$] was found in both a wild type *RNQ1* and an *rnq1:HIS3* segregant.

To determine if the unspecified [*PSI*$^+$] phenotype could be created by mixing strong and weak [*PSI*$^+$] propagons in a single cell, we micromanipulated zygotes resulting from the mating of strong and weak [*PSI*$^+$] and examined their progeny. These zygotes were allowed to grow for 17 or 72 hrs and the resulting microcolonies were plated on YPD (Fig. 15). Among the 76 individual zygotes and their diploid progeny examined, strong [*PSI*$^+$] prevailed for 53 zygotes while weak [*PSI*$^+$] prevailed in the remaining 23 zygotes. SDD-AGE analysis of progeny from representative zygotes showed that the pink and white zygote progeny respectively had oligomer sizes similar to weak and strong [*PSI*$^+$] parents. There were <1% pink colonies among white zygote populations and <1% white colonies among pink zygote populations. On subculturing, the colors of pink and white colored colonies remain unchanged. These results suggest that all zygotes arising from crosses of weak and strong [*PSI*$^+$] cells are not identical. Possibly, depending upon the numbers of propagons in the particular mating cells, weak [*PSI*$^+$] can sometime take over the population. Previously, we found only strong [*PSI*$^+$] to prevail in such crosses, but different variants were used and only 8 diploid colonies were examined in the earlier study (Bradley *et al.*, 2002). In any event, none of the progeny from any of the zygotes showed sectored colonies characteristic of unspecified [*PSI*$^+$].

To further compare cells that contain a mixture of weak and strong [*PSI*$^+$] propagons with unspecified [*PSI*$^+$] we examined the types of propagons present

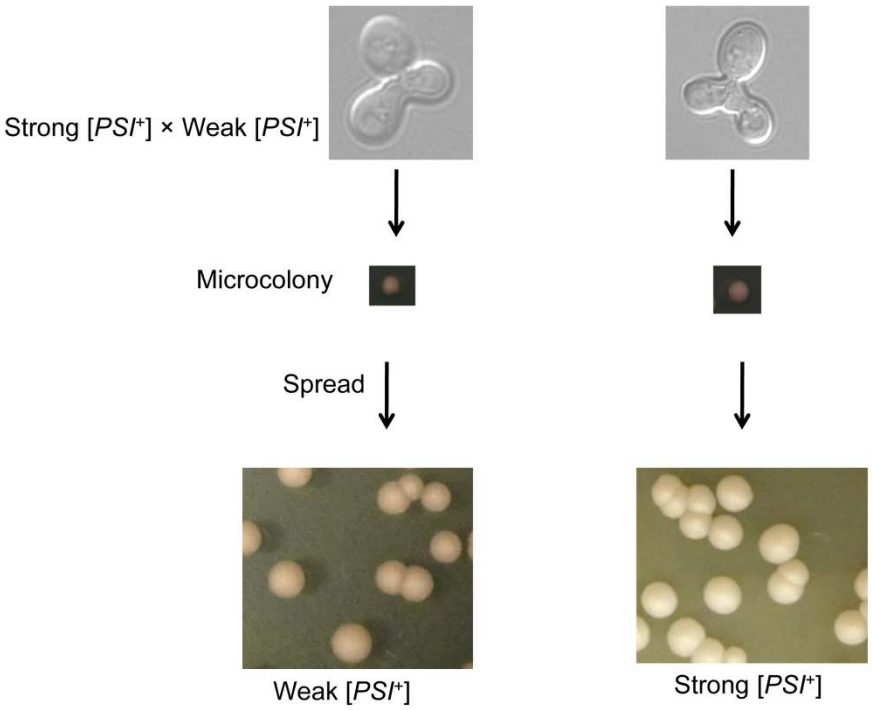

Figure 15. A mixture of two [*PSI*$^+$] variants does not mimic unspecified [*PSI*$^+$]. **A.** Strong [*PSI*$^+$] and weak [*PSI*$^+$] were mated and zygotes were micromanipulated individually. Zygotes were allowed to grow to form microcolonies before being spread on YPD plates to determine the variant present in the progeny cells. Some zygotes gave rise to essentially all strong [*PSI*$^+$] while other zygotes gave rise to essentially all weak [*PSI*$^+$] progeny.

in these two types of cells. We grew individual cells from an unspecified [*PSI*$^{+}$] culture, as well as zygotes formed by mating weak and strong [*PSI*$^{+}$] haploids, into colonies on medium containing GuHCl. GuHCl, which does not block cell division, inhibits the shearing of prion aggregates or fibers, thereby stopping propagons from dividing (Eaglestone *et al.*, 2000). Thus only the propagons already present in the initial cell or zygote are distributed to her progeny. Therefore, theoretically as the colony grows, cells equivalent to the number of propagons in the original cell or zygote should each inherit a single propagon while the rest of the cells in the colony will not get any propagons (Cox *et al.*, 2003). Only the cells containing a propagon will become [*PSI*$^{+}$] when grown on YPD without GuHCl and the [*PSI*$^{+}$] variant present will reflect the variant of the inherited propagon. The type and number of [*PSI*$^{+}$] colonies obtained after plating the colonies grown in the presence of GuHCl on YPD, corresponds to the propagon type and number present in the initial cell or zygote when it was first put on GuHCl.

Twenty cells isolated from unspecified [*PSI*$^{+}$] colonies were grown into microcolonies on 3 mM GuHCl for 72 hrs and then spread on YPD to score the non-red [*PSI*$^{+}$] colonies for their variant type which reflects the type of propagon they inherited from the original cell placed on GuHCl (Eaglestone *et al.*, 2000). This analysis showed that 11 cells contained only unspecified [*PSI*$^{+}$] propagons, 7 had only strong [*PSI*$^{+}$] propagons, 1 had only weak [*PSI*$^{+}$] propagons and 2 contained a

mixture of unspecified, strong and weak [*PSI*$^{+}$] propagons (Fig. 16). Although our propagon count was lower than the propagon number previously reported for other strong and weak [*PSI*$^{+}$] variants (Cox *et al.*, 2003), in agreement with previous findings, we show strong [*PSI*$^{+}$] daughters had a proportionally higher number of propagons than weak [*PSI*$^{+}$] daughters. We also found that the propagon number for unspecified [*PSI*$^{+}$] was around the same as that for weak [*PSI*$^{+}$] (Table 2). In contrast to the above results, analogous experiments on zygotes formed by mating weak and strong [*PSI*$^{+}$] haploids showed that they contained only weak and strong [*PSI*$^{+}$] propagons and never any unspecified propagons (Table 3).

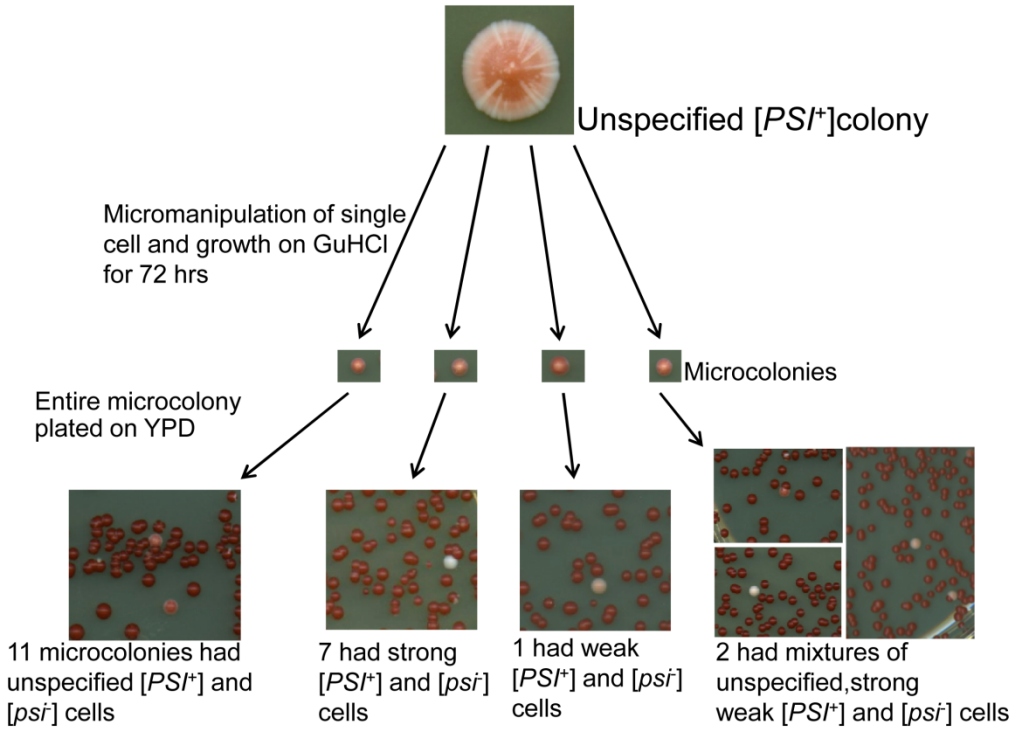

Figure 16. Unspecified [*PSI*$^{+}$] prion propagon analysis. Unspecified [*PSI*$^{+}$] cells were subjected to propagon analysis by micromanipulating individual cells from unspecified [*PSI*$^{+}$] colonies and placing them on medium containing 3mM GuHCl to block their propagons from multiplying. After 72 hrs the whole microcolony was spread on YPD without GuHCl to score for unspecified, weak and strong [*PSI*$^{+}$]. The number of non-red colonies reflected the number of propagons in the original cell.

Table2: Propagon study of 21 cells from an unspecified [*PSI*$^+$] colony

Cells	No. of propagons that are*			Average number of propagons
	Unspecified	Strong	Weak	
1 2 3 4 5 6 7 8 9 10 11	10 14 21 19 33 22 13 11 17 51 68	0 0 0 0 0 0 0 0 0 0 0	0 0 0 0 0 0 0 0 0 0 0	Unspecified [*PSI*$^+$]~26
12 13 14 15 16 17 18	0 0 0 0 0 0 0	156 113 89 116 39 4 19	0 0 0 0 0 0 0	Strong [*PSI*$^+$] ~77
19	0	0	22	Weak [*PSI*$^+$]~22
20 21	14 5	5 1	2 2	Mixture of all 3 ~14

*The numbers of unspecified , strong and weak [*PSI*$^+$] propagons present in each of 21 cells from an unspecified [*PSI*$^+$] culture were determined as described previously (Cox *et al.*, 2003).

Table 3: Propagon counts of strong [*PSI*$^+$] × weak [*PSI*$^+$] zygotes.

Strong [*PSI*$^+$] × Weak [*PSI*$^+$] zygotes	No. of propagons in zygote that are*			Average number of propagons
	Unspecified	Strong	Weak	
1.	0	873	125	Strong [*PSI*$^+$]~554
2.	0	262	99	Weak [*PSI*$^+$]~115
3.	0	638	155	Unspecified [*PSI*$^+$]=0
4.	0	445	93	
5.	0	443	102	
6.	0	668	140	
7.	0	498	120	
8.	0	602	85	

*The numbers of unspecified , strong and weak [*PSI*$^+$] propagons present in each of the eight zygotes were determined as described previously (Cox *et al.*, 2003).

E. Discussion:

[*PSI*$^{+}$] induction by overexpression of Sup35NM-GFP is associated with the transient appearance of ring or line-like aggregates (Zhou *et al.*, 2001) and causes the appearance of variants of [*PSI*$^{+}$] that differ in such things as levels of nonsense suppression, stability, level of Sup35 aggregation and toxicity (Derkatch *et al.*, 1996; Kryndushkin *et al.*, 2003; McGlinchey *et al.*, 2011; Uptain *et al.*, 2001; Zhou *et al.*, 1999). Since, overexpression of Sup35NM-GFP does not cause ring formation and does not alter the variant phenotype in established [*PSI*$^{+}$] cells, it appears that once a prion variant is established it is unaltered by the overexpression of prion protein (Fig. 4). Here, we used the appearance of the ring or line-like aggregates to investigate the process of prion variant establishment. We show that a single [*PSI*$^{+}$] variant is not always specified when ring aggregates are formed (Fig. 2). Rather, we found that the potential to give rise to more than one [*PSI*$^{+}$] variant can co-exist in cells with ring aggregates and can be transmitted to daughter cells.

It is important to keep in mind that we only scored differences between phenotypically distinct strong and weak [*PSI*$^{+}$] strains. Thus while our work proves that multiple variants can be induced in a single cell, we can only detect a small fraction of such events. This is because progeny that are all strong (or all weak) [*PSI*$^{+}$] may still contain distinct variants that differ in more subtle properties.

We considered the possibility that only single [*PSI*$^{+}$] variants appeared in mother cells, but were sometimes lost after being transmitted to a daughter, followed by the induction of a new [*PSI*$^{+}$] variant in the same mother cell that was then transmitted to a later daughter. However, since ring aggregates have never been observed to disappear and then reappear this seems unlikely. It is also unlikely that [*PSI*$^{+}$] could arise *de novo* in daughters who lost, or never inherited, [*PSI*$^{+}$] from their ring cell mothers since daughters do not have high levels of Sup35NM-GFP.

Another novel finding of this work was that 5% of the [*PSI*$^{+}$] prions induced were “unspecified” in variant type. While it has been reported previously that newly appearing [*PSI*$^{+}$] is often unstable, this instability referred to the loss of the prion—not its conversion into another variant (Derkatch *et al.*, 1996). We did previously describe an ‘undifferentiated [*PSI*$^{+}$]’, propagated by the N-domain of Sup35 (in the absence of the M domain, Sup35 1-113, Sup35 1-123), that was capable of forming both strong and weak [*PSI*$^{+}$] when cytoduced into wild-type Sup35 recipients (Bradley and Liebman, 2004). In contrast to this earlier work, the unspecified [*PSI*$^{+}$] described here is propagated by full length Sup35 including the M domain.

One possible explanation for unspecified [*PSI*$^{+}$] is that it is a toxic variant, causing efficient selection for altered non-toxic variants, such as weak or strong

[*PSI*$^{+}$]. However, toxic or lethal [*PSI*$^{+}$] causes excessive nonsense suppression (McGlinchey *et al.*, 2011), while unspecified [*PSI*$^{+}$] has a phenotype intermediate between strong and weak [*PSI*$^{+}$] and is not associated with any reduction in growth.

Thus, to explain our data we consider two other non-exclusive hypotheses 1) that multiple prion variants are induced in a single cell and 2) that a single newly appearing prion aggregate is capable of changing into different variant conformations. The multiple variant hypothesis proposes that cells with initial ring aggregates contain [*PSI*$^{+}$] aggregates with more than one conformation. According to this hypothesis our data could be explained if these different shaped [*PSI*$^{+}$] prion conformers sometimes segregated from each other, and sometimes were transferred simultaneously, when transmitted to daughter cells. However, this hypothesis alone does not explain the data since in contrast to the ring containing cells that give rise to considerable proportions of both strong and weak [*PSI*$^{+}$] progeny, individual zygotes known to contain a mixture of strong and weak [*PSI*$^{+}$] propagons always gave rise to essentially all strong or all weak [*PSI*$^{+}$] progeny. In addition, according to this hypothesis, cells with unspecified [*PSI*$^{+}$] would have to retain both variants indefinitely.

In contrast, we show that cells with the unspecified [*PSI*$^{+}$] generally do not contain a mixture of different prion variant propagons. Indeed, many cells in the unspecified [*PSI*$^{+}$] colony contained only unspecified [*PSI*$^{+}$] propagons, that by definition gave rise to progeny of more than one variant, (see Table 2). This

strongly supports the idea of heritable prion conformations that can frequently undergo different alterations in conformation giving rise to distinct prion variants. We call this phenomenon prion maturation.

The maturation hypothesis proposes that newly formed propagons, carry the dynamic ability to fold into more than one conformation before becoming established as a mature prion with a specific variant shape. In a newly formed [*PSI*$^{+}$] cells, because of their immature state, these aggregates could mature into both strong and weak [*PSI*$^{+}$] variant conformers. The time required for an immature propagon to mature could vary, sometimes occurring in the ring cell itself, sometimes in its daughters or granddaughters—and sometimes persisting indefinitely. Our finding that increasing the time of overexpression of Sup35NM-GFP in [*psi*$^{-}$] [*PIN*$^{+}$] cells, ncreases the proportion of ring cells giving rise to only a single variant *de novo* is consistent with the maturation hypothesis, where the extended period of overexpression could provide the time needed for immature propagons to mature into specified variant conformations.

It seems unlikely that specified- weak and strong propagons present in the mother cell would completely segregate out in daughters and granddaughters since each is likely to inherit many propagons from her mother. Instead, possibly once an immature propagon becomes a specified variant in such daughter or granddaughter cells, the specified propagon could seed the maturation of the remaining unspecified propagons to become specified. However, since individual

unspecified [*PSI*$^{+}$] cells can contain both specified and unspecified propagons, the presence of specified propagon does not always immediately cause the maturation of other co-existing unspecified [*PSI*$^{+}$] variant propagons.

The finding that unspecified propagons exist, as distinct from a mixture of weak and strong [*PSI*$^{+}$] propagons, provides strong support for the maturation model - at least for unspecified [*PSI*$^{+}$]. This together with the fact that the behavior of unspecified [*PSI*$^{+}$] cells is distinct from that of zygotes containing a mixture of strong and weak [*PSI*$^{+}$] propagons supports the hypothesis that immature unspecified propagons are present in unspecified [*PSI*$^{+}$] cells. While it is possible that the unspecified propagons contain a mixture of strong and weak fibers that continue to propagate together, we prefer the hypothesis that the conformation of the unspecified propagons is distinct from both strong and weak [*PSI*$^{+}$]. However, the presence of both weak and strong [*PSI*$^{+}$] in some single unspecified [*PSI*$^{+}$] cells also supports the multiple variant hypothesis. Thus it appears that a combination of both the maturation and multiple variant hypotheses best explain our results.

Prion “adaptation” has been proposed to explain why primary passage of the mammalian PrP prion across species lines is often associated with prolonged incubation periods, while subsequent intra-species propagation results in shorter incubation periods and increased lethality (Bruce *et al.*, 1994; Collinge *et al.*, 1995; Fraser *et al.*, 1992; Hill and Collinge, 2004; Prusiner *et al.*, 1990; Race *et*

al., 2001). Likewise there is a striking difference in the infectious properties of Chronic Wasting Disease prions after multiple rounds of PMCA (Protein Misfolding Cyclic Amplification) or passages in mice when compared to the original inoculum (Meyerett *et al.*, 2008). Cervid PrP^{Sc} can catalyze the conversion of human PrP^{C} to PrP^{Sc} but only after several passages with human PrP^{C} either in transgenic mice or *in vitro (Barria et al., 2011)*. To explain these observations, it has been proposed that foreign infecting PrP protein is in a conformation that is incompatible with propagation of a stable prion when transmitted to the host PrP sequence. Therefore, the host PrP protein will propagate unstable prion conformations until a stable conformation is acquired. Thus, the same host protein sequence goes from an unstable conformation to a stable one resulting in the more efficient conversion of PrP^{c} to the toxic PrP^{Sc} prion species (Collinge and Clarke, 2007; Collinge, 2012). Alterations in the conformation of mammalian PrP prions (called prion “mutations”) have also been proposed to explain this adaptation phenomenon. It is proposed that such mutations result in a prion population composed of a “cloud” of different conformations. Depending upon the suitability of the environment, the conformation that multiplies fastest will take over the others e.g. during sequential passages in a new species (Weissmann, 2012).

Although the species barrier is caused by incompatibility between two PrP sequences, the adaptation phase is very reminiscent of the prion maturation

process hypothesized to explain our results as they both involve evolution of conformations of a single protein (Collinge and Clarke, 2007; Race *et al.*, 2001). It is possible that an “unspecified” conformation first arises in the new host because the new sequence is incompatible with the conformation of the infecting prion, and that adaptation within this species involves the conversion of the “unspecified” conformation into one that is compatible in the new host. This suggests that PrP adaptation may involve an unspecified conformation that can mature into a compatible conformation, as well as a sorting out prions with different conformations.

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F. References:

Alberti, S., Halfmann, R., King, O., Kapila, A., and Lindquist, S. (2009) A systematic survey identifies prions and illuminates sequence features of prionogenic proteins. *Cell* **137**: 146-158.

Bagriantsev, S., and Liebman, S.W. (2004) Specificity of prion assembly in vivo. [PSI+] and [PIN+] form separate structures in yeast. *J Biol Chem* **279**: 51042-51048.

Bagriantsev, S.N., Kushnirov, V.V., and Liebman, S.W. (2006) Analysis of amyloid aggregates using agarose gel electrophoresis. *Methods Enzymol* **412**: 33-48.

Barria, M.A., Telling, G.C., Gambetti, P., Mastrianni, J.A., and Soto, C. (2011) Generation of a new form of human PrP(Sc) in vitro by interspecies transmission from cervid prions. *J Biol Chem* **286**: 7490-7495.

Bradley, M.E., Edskes, H.K., Hong, J.Y., Wickner, R.B., and Liebman, S.W. (2002) Interactions among prions and prion "strains" in yeast. *Proc Natl Acad Sci U S A* **99 Suppl 4**: 16392-16399.

Bradley, M.E., Bagriantsev, S., Vishveshwara, N., and Liebman, S.W. (2003) Guanidine reduces stop codon read-through caused by missense mutations in SUP35 or SUP45. *Yeast* **20**: 625-632.

Bradley, M.E., and Liebman, S.W. (2003) Destabilizing interactions among [PSI(+)] and [PIN(+)] yeast prion variants. *Genetics* **165**: 1675-1685.

Bradley, M.E., and Liebman, S.W. (2004) The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI+] prions. *Mol Microbiol* **51**: 1649-1659.

Bruce, M., Chree, A., McConnell, I., Foster, J., Pearson, G., and Fraser, H. (1994) Transmission of bovine spongiform encephalopathy and scrapie to

mice: strain variation and the species barrier. *Philos Trans R Soc Lond B Biol Sci* **343**: 405-411.

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G., and Liebman, S.W. (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. *Science* **268**: 880-884.

Choe, Y.J., Ryu, Y., Kim, H.J., and Seok, Y.J. (2009) Increased [PSI+] appearance by fusion of Rnq1 with the prion domain of Sup35 in Saccharomyces cerevisiae. *Eukaryot Cell* **8**: 968-976.

Collinge, J., Palmer, M.S., Sidle, K.C., Hill, A.F., Gowland, I., Meads, J., Asante, E., Bradley, R., Doey, L.J., and Lantos, P.L. (1995) Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature* **378**: 779-783.

Collinge, J., and Clarke, A.R. (2007) A general model of prion strains and their pathogenicity. *Science* **318**: 930-936.

Collinge, J. (2012) Cell biology. The risk of prion zoonoses. *Science* **335**: 411-413.

Conde, J., and Fink, G.R. (1976) A mutant of Saccharomyces cerevisiae defective for nuclear fusion. *Proc Natl Acad Sci U S A* **73**: 3651-3655.

Cox, B., Ness, F., and Tuite, M. (2003) Analysis of the generation and segregation of propagons: entities that propagate the [PSI+] prion in yeast. *Genetics* **165**: 23-33.

Cox, B.S., Tuite, M.F., and McLaughlin, C.S. (1988) The psi factor of yeast: a problem in inheritance. *Yeast* **4**: 159-178.

Crow, E.T., and Li, L. (2011) Newly identified prions in budding yeast, and their possible functions. *Semin Cell Dev Biol*.

Derkatch, I.L., Chernoff, Y.O., Kushnirov, V.V., Inge-Vechtomov, S.G., and Liebman, S.W. (1996) Genesis and variability of *[PSI]* prion factors in *Saccharomyces cerevisiae*. *Genetics* **144**: 1375-1386.

Derkatch, I.L., Bradley, M.E., Zhou, P., Chernoff, Y.O., and Liebman, S.W. (1997) Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae. *Genetics* **147**: 507-519.

Derkatch, I.L., Bradley, M.E., Hong, J.Y., and Liebman, S.W. (2001) Prions affect the appearance of other prions: the story of [PIN(+)]. *Cell* **106**: 171-182.

Derkatch, I.L., Uptain, S.M., Outeiro, T.F., Krishnan, R., Lindquist, S.L., and Liebman, S.W. (2004) Effects of Q/N-rich, polyQ, and non-polyQ amyloids on the de novo formation of the [PSI+] prion in yeast and aggregation of Sup35 in vitro. *Proc Natl Acad Sci U S A* **101**: 12934-12939.

Du, Z., Park, K.W., Yu, H., Fan, Q., and Li, L. (2008) Newly identified prion linked to the chromatin-remodeling factor Swi1 in Saccharomyces cerevisiae. *Nat Genet* **40**: 460-465.

Eaglestone, S.S., Ruddock, L.W., Cox, B.S., and Tuite, M.F. (2000) Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [PSI(+)] of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **97**: 240-244.

Fraser, H., Bruce, M.E., Chree, A., McConnell, I., and Wells, G.A. (1992) Transmission of bovine spongiform encephalopathy and scrapie to mice. *J Gen Virol* **73 (Pt 8)**: 1891-1897.

Ganusova, E.E., Ozolins, L.N., Bhagat, S., Newnam, G.P., Wegrzyn, R.D., Sherman, M.Y., and Chernoff, Y.O. (2006) Modulation of prion formation, aggregation, and toxicity by the actin cytoskeleton in yeast. *Mol Cell Biol* **26**: 617-629.

Gietz, R.D., and Woods, R.A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* **350**: 87-96.

Glover, J.R., Kowal, A.S., Schirmer, E.C., Patino, M.M., Liu, J.J., and Lindquist, S. (1997) Self-seeded fibers formed by Sup35, the protein determinant of *[PSI$^+$],* a heritable prion-like factor of *S. cerevisiae*. *Cell* **89**: 811-819.

Hill, A.F., and Collinge, J. (2004) Prion strains and species barriers. *Contrib Microbiol* **11**: 33-49.

Hosoda, N., Kobayashi, T., Uchida, N., Funakoshi, Y., Kikuchi, Y., Hoshino, S., and Katada, T. (2003) Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation. *J Biol Chem* **278**: 38287-38291.

Jung, G., and Masison, D.C. (2001) Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions. *Curr Microbiol* **43**: 7-10.

King, C.Y., Tittmann, P., Gross, H., Gebert, R., Aebi, M., and Wuthrich, K. (1997) Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. *Proc Natl Acad Sci U S A* **94**: 6618-6622.

King, C.Y. (2001) Supporting the structural basis of prion strains: induction and identification of [PSI] variants. *J Mol Biol* **307**: 1247-1260.

King, C.Y., and Diaz-Avalos, R. (2004) Protein-only transmission of three yeast prion strains. *Nature* **428**: 319-323.

Kochneva-Pervukhova, N.V., Chechenova, M.B., Valouev, I.A., Kushnirov, V.V., Smirnov, V.N., and Ter-Avanesyan, M.D. (2001) [Psi(+)] prion generation in yeast: characterization of the 'strain' difference. *Yeast* **18**: 489-497.

Krishnan, R., and Lindquist, S.L. (2005) Structural insights into a yeast prion illuminate nucleation and strain diversity. *Nature* **435**: 765-772.

Kryndushkin, D.S., Alexandrov, I.M., Ter-Avanesyan, M.D., and Kushnirov, V.V. (2003) Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *J Biol Chem* **278**: 49636-49643.

Kushnirov, V.V., Kochneva-Pervukhova, N.V., Chechenova, M.B., Frolova, N.S., and Ter-Avanesyan, M.D. (2000a) Prion properties of the Sup35 protein of yeast Pichia methanolica. *Embo J* **19**: 324-331.

Kushnirov, V.V., Kryndushkin, D.S., Boguta, M., Smirnov, V.N., and Ter-Avanesyan, M.D. (2000b) Chaperones that cure yeast artificial [PSI(+)] and their prion-specific effects. *Curr Biol* **10**: 1443-1446.

Kushnirov, V.V., Alexandrov, I.M., Mitkevich, O.V., Shkundina, I.S., and Ter-Avanesyan, M.D. (2006) Purification and analysis of prion and amyloid aggregates. *Methods* **39**: 50-55.

Liu, J.J., Sondheimer, N., and Lindquist, S.L. (2002) Changes in the middle region of Sup35 profoundly alter the nature of epigenetic inheritance for the yeast prion [PSI+]. *Proc Natl Acad Sci U S A* **99 Suppl 4**: 16446-16453.

Mathur, V., Taneja, V., Sun, Y., and Liebman, S.W. (2010) Analyzing the birth and propagation of two distinct prions, [PSI+] and [Het-s](y), in yeast. *Mol Biol Cell* **21**: 1449-1461.

McGlinchey, R.P., Kryndushkin, D., and Wickner, R.B. (2011) Suicidal [PSI+] is a lethal yeast prion. *Proc Natl Acad Sci U S A* **108**: 5337-5341.

Meyerett, C., Michel, B., Pulford, B., Spraker, T.R., Nichols, T.A., Johnson, T., Kurt, T., Hoover, E.A., Telling, G.C., and Zabel, M.D. (2008) In vitro strain adaptation of CWD prions by serial protein misfolding cyclic amplification. *Virology* **382**: 267-276.

Patel, B.K., Gavin-Smyth, J., and Liebman, S.W. (2009) The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion. *Nat Cell Biol* **11**: 344-349.

Patel, B.K., and Liebman, S.W. (2011) Corrigendum. *Genetics* **188**: 1023.

Prusiner, S.B., Scott, M., Foster, D., Pan, K.M., Groth, D., Mirenda, C., Torchia, M., Yang, S.L., Serban, D., Carlson, G.A., and et al. (1990) Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell* **63**: 673-686.

Prusiner, S.B. (1998) Prions. *Proc Natl Acad Sci U S A* **95**: 13363-13383.

Race, R., Raines, A., Raymond, G.J., Caughey, B., and Chesebro, B. (2001) Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. *J Virol* **75**: 10106-10112.

Rasband, W.S. (1997-2012) ImageJ. *U. S. National Institutes of Health, Bethesda,*
Maryland, USA, http://imagej.nih.gov/ij/.

Roberts, B.E., Duennwald, M.L., Wang, H., Chung, C., Lopreiato, N.P., Sweeny, E.A., Knight, M.N., and Shorter, J. (2009) A synergistic small-molecule combination directly eradicates diverse prion strain structures. *Nat Chem Biol* **5**: 936-946.

Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F.E., and Prusiner, S.B. (1998) Eight prion strains have PrP(Sc) molecules with different conformations. *Nat Med* **4**: 1157-1165.

Satpute-Krishnan, P., and Serio, T.R. (2005) Prion protein remodelling confers an immediate phenotypic switch. *Nature* **437**: 262-265.

Schlumpberger, M., Prusiner, S.B., and Herskowitz, I. (2001) Induction of distinct [URE3] yeast prion strains. *Mol Cell Biol* **21**: 7035-7046.

Sherman, F., Fink, G. R. & Hicks, J. B. (1986) *Methods in Yeast Genetics.* Plainview, New York: Cold Spring Harbor Lab.

Sondheimer, N., and Lindquist, S. (2000) Rnq1: an epigenetic modifier of protein function in yeast. *Mol Cell* **5**: 163-172.

Suzuki, G., Shimazu, N., and Tanaka, M. (2012) A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress. *Science* **336**: 355-359.

Tanaka, M., Chien, P., Naber, N., Cooke, R., and Weissman, J.S. (2004) Conformational variations in an infectious protein determine prion strain differences. *Nature* **428**: 323-328.

Tanaka, M., Collins, S.R., Toyama, B.H., and Weissman, J.S. (2006) The physical basis of how prion conformations determine strain phenotypes. *Nature* **442**: 585-589.

Ter-Avanesyan, M.D., Kushnirov, V.V., Dagkesamanskaya, A.R., Didichenko, S.A., Chernoff, Y.O., Inge-Vechtomov, S.G., and Smirnov, V.N. (1993) Deletion analysis of the *SUP35* gene of the yeast *Saccharomyces cerevisiae* reveals two non-overlapping functional regions in the encoded protein. *Mol Microbiol* **7**: 683-692.

Toyama, B.H., Kelly, M.J., Gross, J.D., and Weissman, J.S. (2007) The structural basis of yeast prion strain variants. *Nature* **449**: 233-237.

Tuite, M.F., Mundy, C.R., and Cox, B.S. (1981) Agents that cause a high frequency of genetic change from *[psi$^+$]* to *[psi$^-$]* in *Saccharomyces cerevisiae*. *Genetics* **98**: 691-711.

Tuite, M.F., and Cox, B.S. (2003) Propagation of yeast prions. *Nat Rev Mol Cell Biol* **4**: 878-890.

Tyedmers, J., Treusch, S., Dong, J., McCaffery, J.M., Bevis, B., and Lindquist, S. (2010) Prion induction involves an ancient system for the sequestration of aggregated proteins and heritable changes in prion fragmentation. *Proc Natl Acad Sci U S A* **107**: 8633-8638.

Uptain, S.M., Sawicki, G.J., Caughey, B., and Lindquist, S. (2001) Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion. *Embo J* **20**: 6236-6245.

Vishveshwara, N., Bradley, M.E., and Liebman, S.W. (2009) Sequestration of essential proteins causes prion associated toxicity in yeast. *Mol Microbiol* **73**: 1101-1114.

Weissmann, C. (2012) Mutation and selection of prions. *PLoS Pathog* **8**: e1002582.

Wickner, R.B. (1994) [URE3] as an altered URE2 protein: evidence for a prion analog in *Saccharomyces cerevisiae*. *Science* **264**: 566-569.

Wickner, R.B., Masison, D.C., and Edskes, H.K. (1995) [PSI] and [URE3] as yeast prions. *Yeast* **11**: 1671-1685.

Zhou, P., Derkatch, I.L., Uptain, S.M., Patino, M.M., Lindquist, S., and Liebman, S.W. (1999) The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. *Embo J* **18**: 1182-1191.

Zhou, P., Derkatch, I.L., and Liebman, S.W. (2001) The relationship between visible intracellular aggregates that appear after overexpression of Sup35 and the yeast prion-like elements [PSI(+)] and [PIN(+)]. *Mol Microbiol* **39**: 37-46.

Chapter III

Exploring the basis of [*PIN*$^{+}$] variant differences in [*PSI*$^{+}$] induction

A. Abstract

Certain soluble proteins can form amyloid-like prion aggregates. Indeed, the same protein can make different types of aggregates, called variants. Each variant is heritable because it attracts soluble homologous protein to join its aggregate, which is then broken into seeds (propagons) and transmitted to daughter cells. [*PSI*$^{+}$] and [*PIN*$^{+}$] are respectively prion forms of Sup35 and Rnq1. Curiously, [*PIN*$^{+}$] enhances the *de novo* induction of [*PSI*$^{+}$]. Different [*PIN*$^{+}$] variants do this to dramatically different extents. Here we investigate the mechanism underlying this effect. Consistent with a heterologous prion cross-seeding model, different [*PIN*$^{+}$] variants preferentially promoted the appearance of different variants of [*PSI*$^{+}$]. However, we did not detect this specificity *in vitro.* Also, [*PIN*$^{+}$] variant cross-seeding efficiencies were not proportional to the level of Rnq1 coimmunocaptured with Sup35, nor to the number of [*PIN*$^{+}$] propagons characteristic for that variant. This leads us to propose that [*PIN*$^{+}$] variants differ in the cross-seeding quality of their seeds, following the Sup35/ [*PIN*$^{+}$] binding step.

B. Introduction

Prions are infectious protein aggregates capable of self-propagation. In mammals the normal cellular PrP^{C} (Prion Protein), which is α- helical, can fold in a β- sheet rich amyloid form called, PrP^{Sc}, which causes fatal neurodegenerative diseases (Prusiner 1998). PrP^{Sc} is infectious because it converts PrP^{C} into PrP^{Sc} in an autocatalytic fashion (Pan, Baldwin et al. 1993). Curiously, there are different heritable forms of PrP^{Sc}, called strains, which cause neurodegenerative diseases with different characteristics and pathologies (Fraser and Dickinson 1973; Bessen and Marsh 1994; Collinge, Sidle et al. 1996). How these distinct prion strains are generated and how they cause distinct pathologies is unknown.

Amyloid diseases are also associated with the conversion of normally folded protein into amyloid that is deposited in various tissues. For example, Alzheimer's disease is associated with Aβ (Amyloid-β-protein) amyloid, Huntington's with mutant huntingtin amyloid, Parkinson's with α-synuclein amyloid and type II diabetes with insulin amyloid precursor peptide (Soto 2003).

Prions also occur in yeast and fungi where they confer specific heritable phenotypes that are transmitted in a dominant non-Mendelian fashion (Cox 1965; Wickner 1994; Sondheimer and Lindquist 2000; Derkatch, Bradley et al. 2001; Du, Park et al. 2008; Alberti, Halfmann et al. 2009; Patel, Gavin-Smyth et al. 2009). Analogous to the mammalian PrP strains, individual yeast prion proteins can fold in multiple heritable conformations that cause distinct differences in the

degree of the altered phenotypes they cause (Derkatch, Chernoff et al. 1996; Schlumpberger, Prusiner et al. 2001; Bradley, Edskes et al. 2002; Krishnan and Lindquist 2005; Tanaka, Collins et al. 2006).

For both the yeast and PrP proteins, a subregion, called the prion domain, is required for prion maintenance and propagation (Oesch, Westaway et al. 1985; Ter-Avanesyan, Kushnirov et al. 1993; Masison and Wickner 1995; Derkatch, Chernoff et al. 1996). Most known yeast prion domains, but not PrP, are rich in glutamines (Q) and/or asparagines (N) (Cohen and Prusiner 1998; Wickner 2004; Du, Park et al. 2008; Alberti, Halfmann et al. 2009; Patel, Gavin-Smyth et al. 2009). Although there is no sequence similarity or functional resemblance between the yeast prions and the mammalian PrP^{Sc} prion, they share the properties of being in β-sheet rich, amyloid-like and protease resistant aggregates (Tuite, Marchante et al. 2011; Liebman and Chernoff 2012; Wickner, Edskes et al. 2012).

Once a protein forms an amyloid “seed”, soluble molecules of the same protein join the amyloid fiber ends and are converted into the seed’s conformation. For a prion to be inherited in yeast, a variety of chaperones, including Hsp104 and Sis1 are required to sheer the amyloid aggregate (Kryndushkin, Alexandrov et al. 2003; Shorter and Lindquist 2004; Higurashi, Hines et al. 2008; Tipton, Verges et al. 2008). This creates more ends available to bind and convert soluble protein and cuts the aggregate into pieces that can

be transmitted to daughter cells (Tuite and Serio 2010). Indeed, deletion of Hsp104 eliminates the propagation of most known yeast prions (Chernoff, Lindquist et al. 1995; Derkatch, Bradley et al. 1997; Moriyama, Edskes et al. 2000; Sondheimer and Lindquist 2000; Derkatch, Bradley et al. 2001; Du, Park et al. 2008; Patel, Gavin-Smyth et al. 2009), as does growth in the presence of low levels of guanidine hydrochloride (GuHCl) that inactivates Hsp104 (Ferreira, Ness et al. 2001; Jung and Masison 2001).

Two of the best studied yeast prions [*PSI*$^{+}$] and [*PIN*$^{+}$] (also called [*RNQ*$^{+}$]) are respectively the prion forms of the Sup35 and Rnq1 proteins (Wickner, Masison et al. 1995; Derkatch, Bradley et al. 1997; Sondheimer and Lindquist 2000; Derkatch, Bradley et al. 2001). Sup35 is a translation termination factor, while the function of Rnq1 is unknown (Stansfield, Jones et al. 1995). The prion domain of Rnq1 is Q/N rich and is located in the C-terminal region of the protein (amino acids 132-405) (Sondheimer and Lindquist 2000; Vitrenko, Pavon et al. 2007; Kadnar, Articov et al. 2010). Sup35 is composed of three structural domains: the Q/N rich N-terminal (amino acids 1-123) domain: the middle M-domain (amino acids 124-254) and the C-domain (amino acids 255-685) (Ter-Avanesyan, Kushnirov et al. 1993; Derkatch, Chernoff et al. 1996; Bradley and Liebman 2004). The Sup35N-domain functions in RNA turn-over (Hosoda, Kobayashi et al. 2003) and is necessary and sufficient for prion formation and aggregation (Ter-Avanesyan, Dagkesamanskaya et al. 1994; Derkatch, Chernoff

et al. 1996). The Sup35M-domain is not essential for prion formation or translation termination, but effects the phenotypes and maintenance of [*PSI*$^{+}$] variants (Liu, Sondheimer et al. 2002; Bradley and Liebman 2004). The Sup35C-domain is essential for translation termination and thus for cell viability (Ter-Avanesyan, Kushnirov et al. 1993). When in the prion form, Sup35 has reduced translation termination factor activity and this causes some read through of nonsense mutations (Cox 1965; Cox, Tuite et al. 1988; Wickner 1994).

Different heritable variants of [*PSI*$^{+}$] were originally distinguished on the basis of their efficiency of nonsense suppression. This is conveniently assayed using the *ade1-14* nonsense mutation, which causes normally white yeast to become red in the absence of suppression. The color can be restored to white in proportion to the level of read through of the *ade1-14* premature nonsense codon (Chernoff, Lindquist et al. 1995; Zhou, Derkatch et al. 1999). In *ade1-14* cells, variants called strong [*PSI*$^{+}$] were white in color because they caused efficient aggregation of Sup35 and thus efficient nonsense suppression. Variants called weak [*PSI*$^{+}$] were pink in color because they had less efficient aggregation of Sup35 and thus nonsense suppression. Also, weak [*PSI*$^{+}$] showed reduced mitotic stability relative to strong [*PSI*$^{+}$], in the presence (Bradley and Liebman 2003) and absence (Derkatch, Chernoff et al. 1996; Derkatch, Bradley et al. 1997; Uptain, Sawicki et al. 2001) of [*PIN*$^{+}$]. Although [*PSI*$^{+}$] variants are generally categorized as strong or weak, there is a continuum of variants with

different levels of nonsense suppression varying from very weak to very strong [*PSI*$^{+}$] (Kochneva-Pervukhova, Chechenova et al. 2001).

[*PSI*$^{+}$] variants differ in the size of SDS-resistant Sup35 polymers. Stronger variants contain smaller polymers than weaker variants (Kryndushkin, Alexandrov et al. 2003), presumably because they are more susceptible to fragmentation by Hsp104 (Tanaka, Collins et al. 2006; Alexandrov, Vishnevskaya et al. 2008). Since variants with smaller Sup35 polymer contain more fiber ends, they more efficiently recruit soluble Sup35 resulting in reduced amounts of functional Sup35.

The examination of other criteria, such as responses to chaperones, efficiency of transmission of [*PSI*$^{+}$] to [*psi*$^{-}$] cells with certain Sup35 mutations or to cells with Sup35 encoded by other species, (King 2001; Tanaka, Collins et al. 2006; Cox, Byrne et al. 2007; Toyama, Kelly et al. 2007; DiSalvo, Derdowski et al. 2011; Verges, Smith et al. 2011) has recently shown that [*PSI*$^{+}$] variants with the same color are not necessarily identical (Lin, Liao et al. 2011; Toyama and Weissman 2011). Nonetheless, the color assay continues to allow a simple distinction between different types of [*PSI*$^{+}$] variants.

Overexpression of the prion domain of Sup35 induces *de novo* [*PSI*$^{+}$] formation presumably because the increased concentration of the Sup35 prion domain makes it more likely that some of the domain molecules will misfold and seed continued conversion. The frequency of this induction of [*PSI*$^{+}$] is

dramatically increased by the presence of another prion, e.g. [*PIN*$^+$] (Derkatch, Bradley et al. 1997). However, once [*PSI*$^+$] is established, [*PIN*$^+$] can be lost and [*PSI*$^+$] continues to propagate (Derkatch, Bradley et al. 2000).

One idea to explain how [*PIN*$^+$] enhances the appearance of [*PSI*$^+$] is that the [*PIN*$^+$] prion binds to and inactivates cellular factors that would otherwise inhibit *de novo* [*PSI*$^+$] prion formation (Osherovich and Weissman 2001). Currently there is no evidence to support this hypothesis. Rather, several lines of evidence support a heterologous cross-seeding mechanism in which the [*PIN*$^+$] prion enhances the appearance of [*PSI*$^+$] by providing an inefficient template for the conversion of soluble Sup35 monomers into amyloid (Derkatch, Bradley et al. 2001).

The heterologous cross-seeding hypothesis is supported by the findings that the efficiency of *in vitro* Sup35NM polymerization into amyloid is enhanced by the addition of heterologous amyloid fiber, e.g. Rnq1 (Derkatch, Uptain et al. 2004; Vitrenko, Gracheva et al. 2007). Furthermore, in such experiments the newly appearing Sup35NM fibers were seen by electron microscopy to emerge from globular aggregates of the Rnq1prion domain (Vitrenko, Gracheva et al. 2007). Also, newly appearing [*PSI*$^+$] aggregates induced and visualized *in vivo* with overexpressed Sup35NM-YFP always overlapped with [*PIN*$^+$] aggregates visualized with overexpressed Rnq1-CFP. However, the overexpression of Rnq1-CFP undoubtedly changed the [*PIN*$^+$] aggregates. Indeed, even in cells with

established [*PSI*$^{+}$] there was often, although not always, overlap between the overexpressed Sup35NM-YFP and Rnq1-CFP aggregates (Derkatch, Uptain et al. 2004). In contrast, in cells with no overexpression, established [*PSI*$^{+}$] and [*PIN*$^{+}$] prions appear to form separate structures lacking a tight interaction (Bagriantsev and Liebman 2004), while Rnq1 did copurify with SDS resistant Sup35 polymers in [*PIN*$^{+}$][*psi*$^{-}$] cells overexpressing Sup35, but not Rnq1 (Salnikova, Kryndushkin et al. 2005). Finally, [*PSI*$^{+}$] appears rapidly *de novo* in [*PIN*$^{+}$] cells expressing low levels of a fusion of the Rnq1 and Sup35 prion domains (Choe, Ryu et al. 2009). Since the Rnq1 domain of the fusion efficiently joins the [*PIN*$^{+}$] aggregate, it also efficiently brings the fused Sup35 prion domain to the vicinity of the [*PIN*$^{+}$] aggregate, which was proposed to promote heterologous cross-seeding. In addition, several mutations in the prion domain of Rnq1 can maintain the [*PIN*$^{+}$] prion, but reduce its ability to promote the induction of [*PSI*$^{+}$]. This suggests a direct Rnq1/Sup35 interaction through the mutated sites (Bardill and True 2009).

Heterologous cross-seeding of amyloids also appears to be a risk factor for human diseases. Mice injected with amyloid-like fibrils made of a variety of peptides, promoted the formation of amyloid protein A, which is associated with systemic AA amyloidosis (Johan et al. 1998). Heterologous cross-seeding has also been observed between the Alzheimer-associated Aβ peptide and the islet amyloid polypeptide (O'Nuallain, Williams et al. 2004), and between Aβ and PrP

(Morales, Estrada et al. 2010). Also, Aβ fibers appear to induce the amyloidosis of tau (Gotz, Chen et al. 2001; Lasagna-Reeves, Castillo-Carranza et al. 2010).

Like PrP and [*PSI*$^{+}$], the [*PIN*$^{+}$] prion exists in different heritable forms. These [*PIN*$^{+}$] variants have been distinguished on the basis of their ability to induce [*PSI*$^{+}$] and the relative amount of aggregated vs. soluble Rnq1 protein present. High [*PIN*$^{+}$] induces [*PSI*$^{+}$] with the highest frequency and contains the least soluble Rnq1, whereas low [*PIN*$^{+}$] induces with the lowest efficiency and contains more soluble Rnq1. However, surprisingly very high [*PIN*$^{+}$], the variant with the highest [*PSI*$^{+}$] induction efficiency contains the most soluble Rnq1 (Bradley, Edskes et al. 2002).

Here we investigated the effects of [*PIN*$^{+}$] variant differences on the induction of [*PSI*$^{+}$]. In support of the heterologous cross-seeding model, different [*PIN*$^{+}$] variants preferentially promoted the *in vivo* appearance of specific variants of [*PSI*$^{+}$]. However, there was no specificity *in vitro*, suggesting the critical involvement of cellular factors. Also, [*PIN*$^{+}$] variants did not differ in their ability to bind to Sup35, nor did differences in their propagon numbers reflect their different phenotypes. Rather, it appears that the [*PIN*$^{+}$] variants differ in the cross-seeding quality of their seeds.

C. Materials and methods

Yeast strains, plasmids and media

The yeast strains used in this study are listed in Table 1. Many are derivatives of 74-D694 (*MAT**a** ade1-14 ura3-52 leu2-3, 112 trp1-289 his3-200*) (Chernoff, Derkach et al. 1993). Yeast was grown at 30°C unless otherwise indicated. Media was as described previously (Sherman 1986). YPD is a complex medium that contains 2% dextrose and is used to grow yeast and distinguish yeast prion variants. Synthetic dextrose (SD) medium lacks one or more amino acid (e.g. medium lacking leucine is called –Leu) to select for appropriate plasmids, or to select for diploid strains. YPG is a complex medium that contains 3% glycerol and is used to select against mitochondrial mutants (petites). To express genes under the *GAL* inducible promoter, 2% raffinose and 2% galactose was used as the sugar source. YPD containing 5 mM GuHCl was used for [*PSI*$^{+}$] curing experiments. Plasmid, p*Cup1-SUP35NM-GFP* (p1182, *CEN2 LEU2*), containing a copper inducible promoter and the prion and middle domains of Sup35 (Sup35NM) fused to green fluorescent protein was used to induce [*PSI*$^{+}$] in various [*PIN*$^{+}$] backgrounds (Zhou, Derkatch et al. 2001).The 2 µ plasmid p*GAL::SUP35C* (p1598) carries Sup35C under the *GAL* promoter and the *TRP 1*selective marker (Vishveshwara, Bradley et al. 2009). Plasmid p*Cup1::RNQ1GFP, HIS3 (*pID116; kindly supplied by Irina Derkatch) was used to

Table 1: Yeast strains used in this study.

Strains	Description	Source
L1749	*MAT**a** ade1-14 ura3-52 leu2-3, 112 trp1-289 his3-200* [*psi*$^-$] High [*PIN*$^+$]	(Derkatch, Bradley et al. 1997)
L1943	*MAT**a** ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200* [*psi*$^-$] Low [*PIN*$^+$]	(Bradley, Edskes et al. 2002)
L1953	*MAT**a** ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200* [*psi*$^-$] Very high [*PIN*$^+$]	(Bradley, Edskes et al. 2002)
L2910	*MAT**a** ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200* [*psi*$^-$][*pin*$^-$]	(Derkatch, Bradley et al. 1997)
L2731	*MAT**a** ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200 HIS3-Gal::SUP35* [*psi*$^-$][*pin*$^-$]	(Vishveshwara, Bradley et al. 2009)
GF837	*MAT**a** ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200,sup35::RMC*	(Bardill and True 2009)
GF840	*MATα ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200 ,rnq1::KanMx4* [*psi*$^-$] High [*PIN*$^+$]	(Bardill and True 2009)
L2642	*MATα ade1-14 ura3-52 leu2-3,112 trp1-289 lys9-A21* [*psi*$^-$][*pin*$^-$]	(Derkatch, Bradley et al. 1997)
GF585	*MATα ade1-14 trp1-289 his3-delta200 ura3-52 leu2-3, 112* [*psi-*][*pin*$^-$]	(Chernoff, Lindquist et al. 1995)

express wild-type Rnq1-GFP to stain [*PIN*$^+$] aggregates in the Rnq1 decoration assay (see below). Plasmid p*RNQ*::*RNQ1*-N297S (p1862) carrying the Rnq1 mutation, N297S, at the C-terminal end and the *HIS3* selective marker in a pRS313 backbone was a kind gift from H. True (Bardill and True 2009). The N297S mutation considerably reduces the efficiency with which [*PIN*$^+$] facilitates the induction of [*PSI*$^+$]. His tagged proteins *His$_{10}$-RNQ1PD* (amino acids 132–405) and *His$_{10}$-SUP35NM* (amino acids 1-254) were, respectively expressed in plasmids p1510 and p1195, with the pJC45 expression vector backbone (Clos and Brandau 1994; Serio, Cashikar et al. 1999; Patel and Liebman 2007). Wild type *RNQ1PD* (131-405) was amplified from p1415 (p*Cup1-RNQ1::GFP*), cut with BamH1 and SacI and ligated into p*Cup1-GFP*, *URA3* (p984) (Derkatch, Bradley et al. 1998) also cut with BamH1 and SacI to create p*Cup1:His$_{10}$ RNQ1PD GFP StrepII* (p1866) with a *URA3* marker.

[*PSI*$^+$] color assay

All yeast strains used in this study have the *ade1-14* allele that has a nonsense mutation and is frequently used to score for [*PSI*$^+$] (Chernoff, Lindquist et al. 1995). In [*psi*$^-$] cells Sup35 functions as a translation termination factor essential for terminating translation at stop codons including the premature *ade1-14* nonsense codon. This causes *ade1-14* [*psi*$^-$] cells to be Ade$^-$ and to accumulate red pigment on rich medium like YPD. In contrast, in [*PSI*$^+$] cells, where Sup35 is aggregated and is not fully functional, there is occasional read

through of the *ade1-14* premature stop codon, hence some full length Ade1 is synthesized giving Ade$^+$ non-red colonies. When the *ade1-14* mutation is efficiently suppressed, e.g. by strong [*PSI*$^+$], cells return to their white color, while weak [*PSI*$^+$] causes inefficient suppression and a pink colony color. We confirmed that the presence of p*GAL::SUP35C* plasmid did not alter the colony color.

[*PSI*$^+$] Sup35NM-GFP and [*PIN*$^+$] Rnq1-GFP decoration assays

When Sup35NM-GFP is expressed in [*PSI*$^+$] cells it decorates the [*PSI*$^+$] aggregates and can be seen as fluorescent dots within a few hours. In contrast, in [*psi*$^-$] cells Sup35NM-GFP remains diffuse (Zhou, Derkatch et al. 2001). Cells to be scored for [*PSI*$^+$] by this assay are sometimes directly transformed with the p*Cup1-SUP35NM-GFP* plasmid and other times are crossed with a [*psi*$^-$][*pin*$^-$] strain bearing the plasmid. Transformants or diploids were examined with a fluorescent microscope after overnight growth in 50 µM Cu. Analogous assays were done for [*PIN*$^+$] using Rnq1-GFP.

Preparation of [*PIN*$^+$] variant specific seeds from yeast

[*PIN*$^+$] variant specific seeds were prepared by inducing expression of tap tagged Rnq1with 50 µM copper in [*PIN*$^+$] and [*pin*$^-$] cells transformed with p*CUP-HIS$_{10}$RNQ1(132-405)-GFP STREP II*. After overnight induction, cells were harvested and His tagged Rnq1 (132-405) aggregates purified with Ni-NTA

agarose affinity column chromatography (Qiagen) following the manufacturer's native conditions instructions.

Recombinant protein expression and purification

His_{10}Sup35-NM (1-254) was induced in *E coli* (BL21DE3) transformed with p1195 by the addition of 1mM IPTG to cultures growing at 37°C (Serio, Cashikar et al. 1999). The bacteria were lysed by sonicating (Branson) cells for 20 seconds with 1 min incubation in ice 5X in 20 mM Tris-HCl and 8 M urea (pH 8.0). Likewise, His_{10}Rnq1-PD (132-405) was induced in *E. coli* (BL21 AI) transformed with p1510 by the addition of 1 mM IPTG together with 0.15% (w/v) l(+) arabinose (Patel and Liebman 2007). Lysis was as above but in 100 mM NaH_2PO_4, 10 mM Tris-Cl and 8 M urea (pH 8.0).

Both the recombinant proteins were purified from lysed cells with Ni-NTA agarose affinity column chromatography (Qiagen) under denaturing conditions in 8M urea following the manufacturer's instructions and were stored at -80°C.

***In vitro* aggregation of Rnq1 (132-405) and Sup35NM**

Denatured Rnq1 (132-405) stored in 8 M urea was diluted to 40-60 µM in aggregation buffer (100 mM sodium phosphate (pH 7.3), 4M urea, 1M NaCl) (Patel and Liebman 2007; Vitrenko, Gracheva et al. 2007). Thioflavin–T dye stock of 5 mM was added to 125 µl of the reaction mixture in a 96-well plate. The microplate was incubated for up to to 48 hrs in a Spectramax-Max microplate reader at 30ºC. The plate was shaken intermittently for 10 sec every 3 min and

aggregation was monitored by fluorescence using an excitation wavelength of 450 nm and an emission wavelength of 482 nm. The reactions were seeded with 8% (v/v) sonicated (10 seconds at 15% power) seeds purified from cells with the different [*PIN*$^{+}$] variants.

The above protocol was also followed for Sup35NM polymerization, except that level of [*PIN*$^{+}$] seed was increased. Here, 10-50% (v/v) of sonicated (10 seconds at 15% power) preformed [*PIN*$^{+}$] fibers were added to the reaction mixture and fluorescence recorded.

[*PIN*$^{+}$] transfection

The protocol described in (King, Wang et al. 2006) for transfection of Sup35 fibers was modified slightly to allow transfection of *in vitro* made Rnq1 fibers. Yeast (L2642 and GF585) of the same mating type, but with complementary markers were harvested at mid log phase and were separately converted into spheroplasts by lyticase treatment. The two spheroplasts population were incubated with Rnq1fibers and then combined. This mixture was plated on medium that selects for fused cells (–His-Lys). Colonies of the fused cells were first screened for the presence of the [*PIN*$^{+}$] prion by mating each of them with a *MAT*a [*pin*$^{-}$] strain carrying the p*Cup1-RNQ1-GFP* plasmid. The unmated fused cells corresponding to the triploids that showed fluorescent dots after overnight growth in 50 µM Cu for were transformed with the p*Cup1-*

SUP35NM-GFP plasmid to enable scoring of the specific [*PIN*$^+$] variant present *via* the Sup35NM-GFP ring induction method (see below).

Distinguishing [*PIN*$^+$] variants by the frequency of Sup35NM-GFP rings/lines

Overexpression of p*Cup1-SUP35NM-GFP* in a [*PIN*$^+$][*psi*$^-$] background gives rise to cells with ring or line like aggregates indicative of [*PSI*$^+$] induction (Zhou, Derkatch et al. 2001). Furthermore, the frequency of the ring/line aggregates is different for each of the [*PIN*$^+$] variants used here (Bradley, Edskes et al. 2002). After 20 hrs induction of Sup35NM-GFP: [*pin*$^-$] cultures showed essentially no cells with rings/lines; low [*PIN*$^+$] cultures had ~10% of cells with rings/lines; high [*PIN*$^+$] cultures had ~25%rings/lines, and very high [*PIN*$^+$] cultures ~40% rings/lines (See Figure 7).

Distinguishing [*PIN*$^+$] variants on the basis of Rnq1 solubility

The solubility of Rnq1 was assayed essentially as described previously (Sondheimer and Lindquist 2000). [*PIN*$^+$] variants differ in the levels of soluble vs. aggregated Rnq1 present in the cell: very high [*PIN*$^+$] has more soluble RNQ1 than low [*PIN*$^+$], which has more soluble Rnq1 than high [*PIN*$^+$] (See Figure 6) (Bradley, Edskes et al. 2002). To determine the levels of soluble vs. aggregated Rnq1, 50 mls of log phase washed cells were resuspended in 750 µl of lysis buffer (50 mM KCl, 10 mM MgCl2, 50 mM Tris/HCl, pH 7.5 and 5% [wt/vol] glycerol, 1:50 diluted protease inhibitor cocktail [Sigma, St. Louis, MO], and 5

mM PMSF), mixed with 750 µl of 0.5 mm glass beads (Biospec, Bartlesville, OK) and lysed by vortexing at high speed for 1 min/5X, with 1 min cooling on ice after each vortexing. Cell debris was removed by centrifuging twice at 600 g for 1 min. Supernatant and pellet fractions of lysates were separated by centrifugation at 80,000 rpm for 30 min in a Sorvall TLA100.1 rotor at 4ºC. The total, supernatant and pellet (resuspended in 250 µl of lysis buffer) fractions were resolved on 10% polyacrylamide gels which were transferred to a polyvinylidenedifluoride membrane and probed with polyclonal Rnq1 antibody (kind gift from S. Lindquist).

[*PSI*$^+$] transfection and scoring of [*PSI*$^+$] transfectants

Sup35NM fibers were transfected as described previously (Tanaka and Weissman 2006). The *in vitro* made amyloid aggregates of recombinant Sup35NM were transformed into [*psi*$^-$][*pin*$^-$] spheroplasts in the presence of the p*Cup1-SUP35NM-GFP,LEU2* plasmid. Leu$^+$ transformants were patched on YPD medium, grown for 3 days and the color examined to score for [*psi*$^-$] (red), weak [*PSI*$^+$] (pink) and strong [*PSI*$^+$] (white) as described above in "[*PSI*$^+$] color assay". All Leu$^+$ transformants with non-red phenotypes were tested for [*PSI*$^+$] with the SUP35NM-GFP aggregate decoration assay (see above), by growing cells overnight in –Leu with 50 µM copper to induce Sup35NM-GFP. Some transformants were further confirmed to be [*PSI*$^+$] by subcloning them on 5 mM GuHCl to test for GuHCl curability. Red colonies were restreaked on YPD to

confirm they were cured by the reappearance of red colony color (Tuite, Mundy et al. 1981; Bradley, Bagriantsev et al. 2003).

Counting [*PIN*$^+$] Propagons

The method previously used to count [*PSI*$^+$] propagons (Cox et al., 2003) was modified for [*PIN*$^+$]. To easily score for [*PIN*$^+$] we used strains carrying the RRP Rnq1 prion domain fused to Sup35MC. In the absence of [*PIN*$^+$] these strains are red. However, in the presence of [*PIN*$^+$] they are white or pink on –Ade, indicative of nonsense suppression (Bardill and True 2009). Individual RRP cells with the different [*PIN*$^+$] variants were micromanipulated from log phase cultures to a 2% noble agar pad that was then placed on a YPD plate containing 3 mM GuHCl and grown for 48 hours. The whole colony was then cut out of the pad, suspended in water and spread on YPD where the number of non-red colonies was taken as the propagon number in the initial cell.

Immunoprecipitation

For the pull down assays the μ MACS protein MicroBeads isolation kit was used (Miltenyibiotec, Auburn, CA). Reactions containing 0.25-1μg/μl of sample protein were incubated with or without anti-GFP antibody for 2 hrs. Then 50 μl of protein G labeled micro beads (Miltenyi Biotec, Auburn, CA) was added and the reactions were mixed and incubated for 30 min on ice. Samples were loaded on magnetic separation μ Columns (Miltenyi Biotec, Auburn, CA), washed 5X's with buffer and eluted in heated 1X sample buffer containing 2% SDS (Bagriantsev,

Gracheva et al. 2008). Eluate (30-40 µl) was run on 10% polyacrylamide gels (Bio-Rad), transferred to PVDF membrane (Bio-Rad), and probed for specific proteins. Anti-GFP antibody was from Roche Applied Science (Indianapolis, IN). Anti-Sup35C antibody developed by Dr. V. Prapapanich (BE4 mouse monoclonal antibodies) was used to detect Sup35 protein. Anti-Rnq1 antibody was a kind gift from Dr. S. Lindquist.

D. Results

1. Specific [*PIN*$^+$] variants preferentially seed specific [*PSI*$^+$] variants *in vivo*

The cross-seeding model predicts that [*PIN*$^+$] aggregates act as an inefficient template to cross-seed *de novo* Sup35 aggregation during the initial stages of [*PSI*$^+$] formation. We hypothesized that during this process the pre-existing [*PIN*$^+$] prion seeds might transfer some of their variant specific structure to the heterologous [*PSI*$^+$] prion being cross-seeded. This could cause certain [*PIN*$^+$] variants to seed one [*PSI*$^+$] variant more frequently than another, and this preference might be reversed for a different [*PIN*$^+$] variant.

To test this we asked if specific [*PIN*$^+$] variants preferentially cross-seed specific [*PSI*$^+$] variants. The plan was to induce [*PSI*$^+$] *de novo* by overexpressing Sup35 in the presence of different [*PIN*$^+$] variants. However, since overexpression of Sup35 in the presence of strong [*PSI*$^+$] causes severe toxicity and growth inhibition with less dramatic effects in the presence of weak [*PSI*$^+$] (Vishveshwara, Bradley et al. 2009), we were concerned that there would be a bias against strong [*PSI*$^+$] isolates. The toxicity arises because [*PSI*$^+$] aggregates bind to and sequester an essential protein (Vishveshwara, Bradley et al. 2009). This can be prevented by overexpressing Sup35C, which lacks the prion domain and therefore does not join the prion aggregate, but binds to the essential protein

and retains full function in translation. Thus in our experiments we overexpressed Sup35C during the induction of [*PSI*$^{+}$].

Integrated *GAL::SUP35* and plasmid born *GAL::SUP35C* were induced simultaneously in cells bearing low, high or very high [*PIN*$^{+}$] variants. Cells were then plated and grown on YPD where colony color was used to distinguish strong [*PSI*$^{+}$] (white), weak [*PSI*$^{+}$] (pink) and [*psi*$^{-}$] (red). As noted previously, newly induced [*PSI*$^{+}$] is often unstable, so many of the lighter color colonies had numerous red [*psi*$^{-}$] sectors (Kochneva-Pervukhova, Chechenova et al. 2001; Bradley and Liebman 2003; Sharma and Liebman 2012) (Figure 1). The frequencies of appearance of all non-red colonies including pink, white and sectored colonies were determined (Figure 2). Non-red colonies were tested for their [*PSI*$^{+}$] status using the Sup35NM-GFP aggregate decoration assay by crossing them with a [*psi*$^{-}$] strain carrying p*Cup1-SUP35NM-GFP.* More than 98% showed dots indicating that they were [*PSI*$^{+}$].

Since the pink vs. white color of sectoring colonies could not be definitely distinguished due to the distraction of the many large red sectors, these colonies were subcloned and the variant type(s) determined by looking at the colony colors of non-red daughter cells (Figure 3,4). Although low or very high [*PIN*$^{+}$] variants destabilize weak [*PSI*$^{+}$] (Bradley and Liebman 2003), the level of destabilization did not prevent us from scoring weak [*PSI*$^{+}$] colonies. A minority of sectoring colonies gave rise to both white and pink colonies.

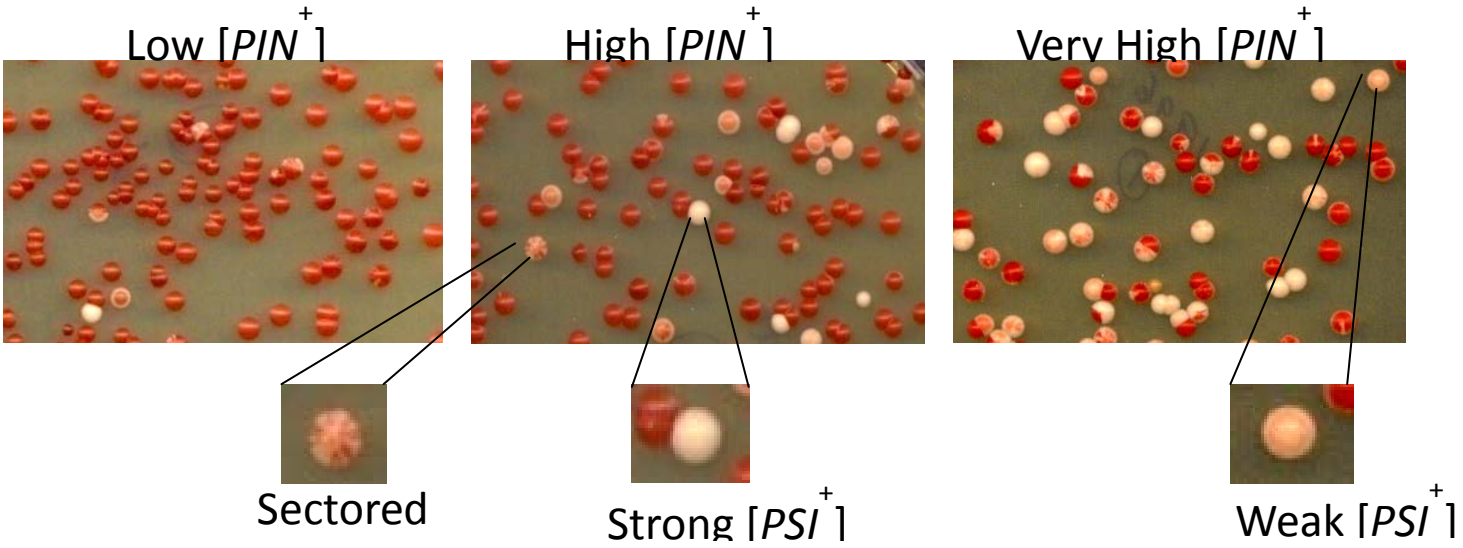


Figure 1. [*PIN*$^{+}$] variants differ in their preference for cross-seeding weak vs. strong [*PSI*$^{+}$]. Appearance of newly induced [*PSI*$^{+}$] colonies in the presence of low, high and very high [*PIN*$^{+}$]. All cells had an integrated copy of *GAL:: SUP35* and a plasmid encoding *GAL:: SUP35C*. Shown are cells plated on YPD following induction of both *SUP35* and *SUP35C* after 4 days of growth on galactose.

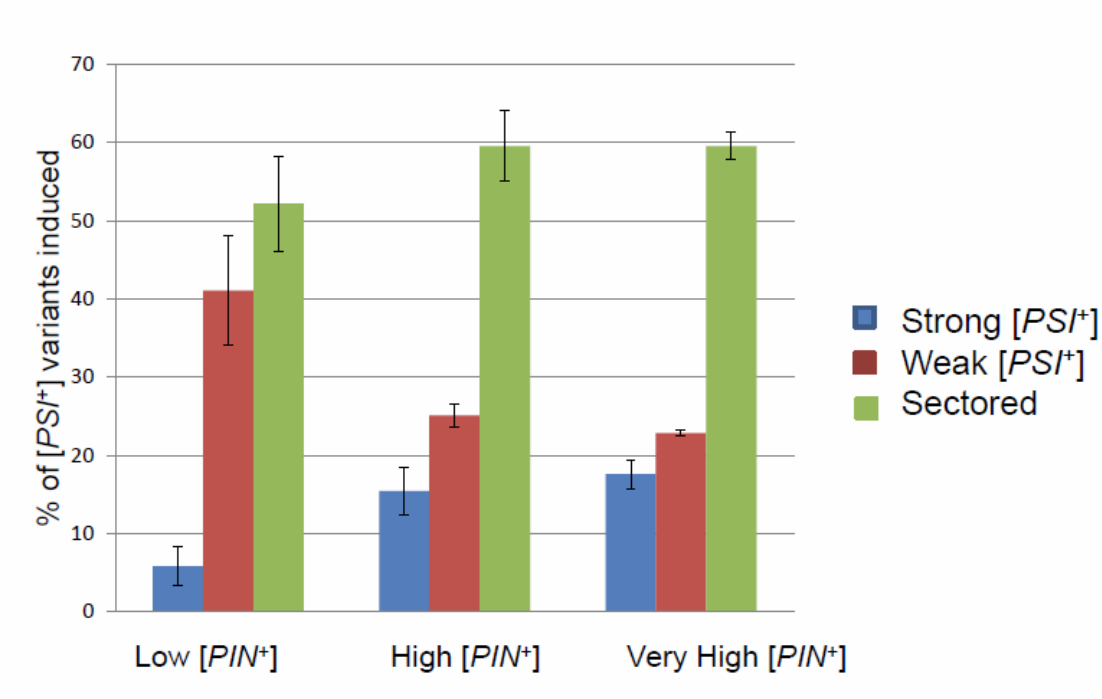


Figure 2. Low, high and very high [*PIN*$^+$] variants give rise to strong [*PSI*$^+$], weak [*PSI*$^+$] and unstable [*PSI*$^+$] (sectored) colonies. The frequencies of white, pink and sectored colonies are shown. Statistical differences ($p<0.0001$) were seen between the distributions of weak and strong [*PSI*$^+$] obtained from low and high [*PIN*$^+$] and from low and very high [*PIN*$^+$].

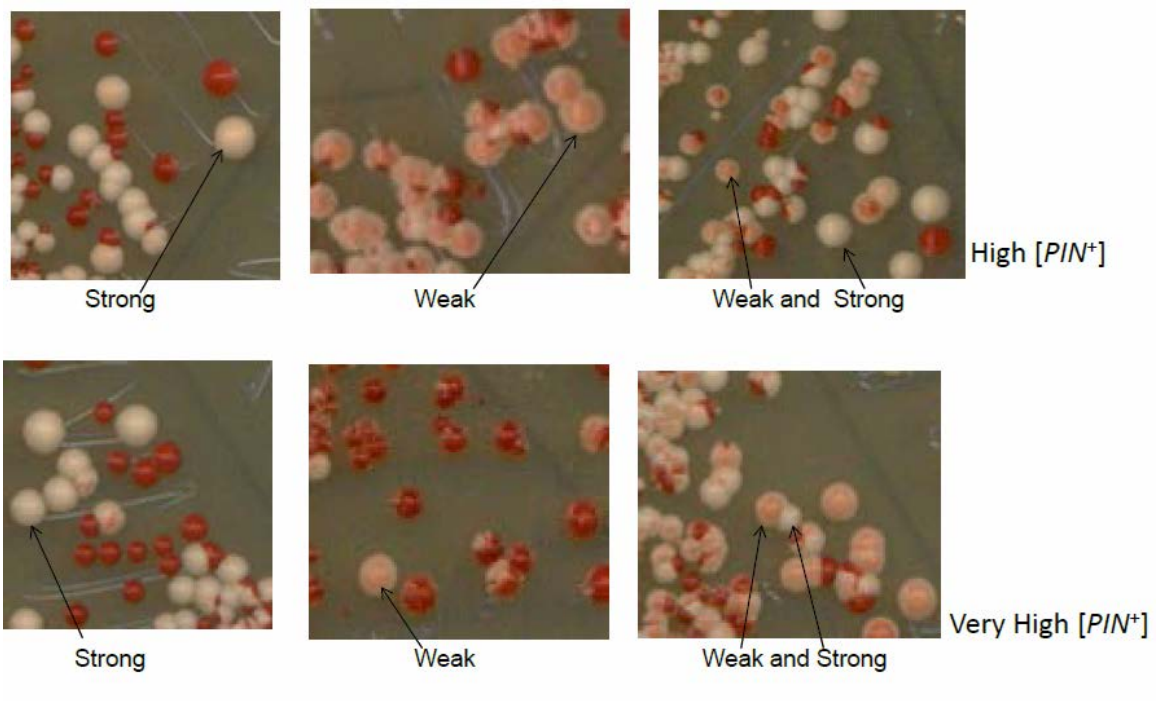


Figure 3. Determination of the variant type of the sectored colonies. The 600 sectored colonies were each subcloned. Six examples are shown. Those giving rise to only red and white colonies were scored as strong [*PSI*$^{+}$]. Those giving rise to only red and pink colonies were scored as weak [*PSI*$^{+}$]. Those giving rise to red, white and pink colonies were scored as mixed.

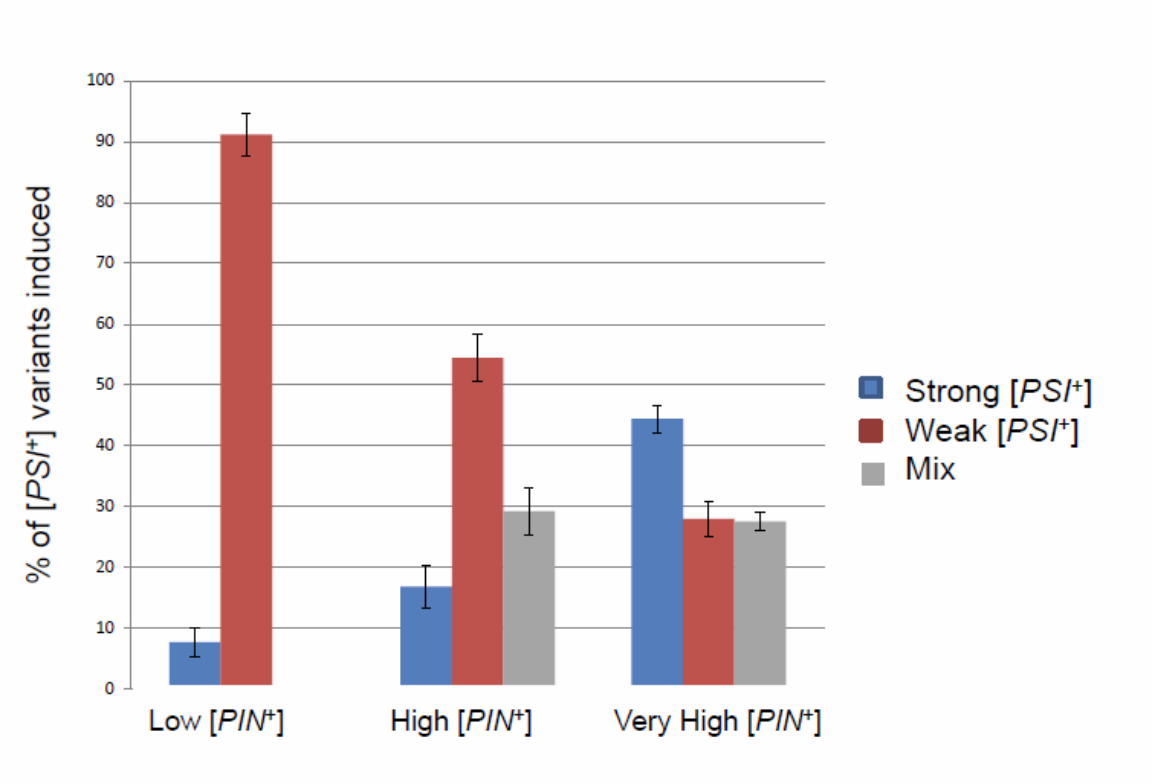


Figure 4. Corrected frequencies of strong and weak [*PSI*$^+$] isolated in the different [*PIN*$^+$] variants. Data in (Figure 2) has been modified to include initially sectoring colonies scored after subcloning as in (Figure 3). Shown is the percent of colonies that were not red obtained from low [*PIN*$^+$] (n= 400), high [*PIN*$^+$] (n= 604) and very high [*PIN*$^+$] (n=1592). Statistical differences ($p < 0.0001$) were seen between the distributions of weak and strong [*PSI*$^+$] obtained from low and high [*PIN*$^+$], from low and very high [*PIN*$^+$] and from high and very high [*PIN*$^+$]. Error bars show the standard error of mean calculated from more than 3 independent experiments. A two by two contingency table and Fisher's exact two tailed test was used to calculate p values.

The data showed that the very high [*PIN*$^+$] variant seeded strong [*PSI*$^+$] more frequently than weak [*PSI*$^+$], while low and high [*PIN*$^+$] seeded weak [*PSI*$^+$] more frequently than strong [*PSI*$^+$].

2.* [*PIN*$^+$] variants make variant specific fibers *in vitro

To determine if cellular factors were involved in the above variant specific *in vivo* result, we asked if the same result could be obtained by cross-seeding recombinant Sup35-NM (1-254) with *in vitro* made low, high and very high [*PIN*$^+$] specific fibers. To do this, the first step was to obtain variant specific [*PIN*$^+$] fibers. Previous work suggested that this should be possible: crude cell extracts from [*PIN*$^+$] variants retained variant specific infectivity when transformed into [*pin*$^-$] yeast (Patel and Liebman 2007); *in vitro* made amyloid like aggregates of Rnq1 (132-405) protein were shown to transform [*pin*$^-$] yeast into [*PIN*$^+$] (Patel and Liebman 2007); Rnq1(132-405) protein that formed fibers *in vitro* at 4ºC vs. 37ºC, gave rise to distinct [*PIN*$^+$] variants when transformed into [*pin*$^-$] yeast (Kalastavadi and True 2010).

To obtain low, high and very high [*PIN*$^+$] specific seed, we purified a STREP-His tap tagged Rnq1 (132-405) construct expressed in the respective [*PIN*$^+$] variant cells (and [*pin*$^-$] as control). These seeds were used to promote the *in vitro* polymerization of recombinant Rnq1 (132-405) protein. The addition of the [*PIN*$^+$] seed significantly shortened the lag phase that proceeded exponential polymerization and led to a larger plateau value (Figure 5). This is consistent with

the nucleated conformational conversion model proposed previously (Serio, Cashikar et al. 2000).

To confirm the variant specificities of these *in vitro* made fibers, they were transfected into [*pin*$^-$] cells where the [*PIN*$^+$] variant present was determined with the Sup35NM-GFP ring aggregate efficiency assay (Materials and Methods) (Table 2). More than 80% of the time the transfectants had the [*PIN*$^+$] variant used to seed the fiber. To confirm this scoring, we analyzed the level of aggregated Rnq1 in at least 10 representative transfectants for each variant type (Figure 6). In each case the [*PIN*$^+$] variant specific ratio of soluble to aggregated Rnq1 protein predicted by the ring scoring method was obtained (Figure 7) (Bradley, Edskes et al. 2002).

3. Variant specific [*PIN*$^+$] fibers cross-seed [*PSI*$^+$] variants

We previously showed that the addition of [*PIN*$^+$] aggregates could increase the efficiency of conversion of soluble recombinant Sup35NM into amyloid *in vitro* (Vitrenko, Gracheva et al. 2007). Here we used the variant specific [*PIN*$^+$] fibers made above to determine whether they would preferentially cross-seed recombinant Sup35NM protein into specific [*PSI*$^+$] variant fibers. Sup35NM fibers, polymerized for 14 hrs with 50% (v/v) of different [*PIN*$^+$] variant specific fiber seeds, were sonicated and transfected onto [*pin*$^-$][*psi*$^-$] yeast. The data show that, the resulting frequencies of strong and weak [*PSI*$^+$] transfectants

Table 2: Formation of variant specific [*PIN*$^+$] fibers

Rnq1 fibers seeded by [*PIN*$^+$] variants	Low [*PIN*$^+$]	High [*PIN*$^+$]	Very High [*PIN*$^+$]
% of [*PIN*$^+$] transfectants with the phenotype of the [*PIN*$^+$] variant used as seed.	80% (n=24)[a]	84% (n=38)[b]	88% (n=25)[c]

Rnq1 (132-405) fibers made with the indicated [*PIN*$^+$] variant specific seeds were transfected into [*pin*$^-$][*psi*$^-$] cells using the cell fusion protocol (King, Wang et al. 2006). Fused cells were scored for [*PIN*$^+$] by the Rnq1-GFP decoration and Sup35NM-GFP ring/ line assays. The numbers of [*PIN*$^+$] transfectants with each [*PIN*$^+$] variant as determined with the Sup35NM-GFP rings/lines frequency assay is shown. Most of the transfectants showed the variant specificity of the seed that was used to make [*PIN*$^+$] fibers.

a=19 of 24 transfectants contained Sup35NM-GFP rings at a rate characteristic of low [*PIN*$^+$] (an average of 9.26 % of the cells had rings); 5 of 24 caused more cells (an average of 20.8 %) to have rings than expected of low [*PIN*$^+$].

b= 32 of 38 [*PIN*$^+$] transfectants contained Sup35NM-GFP rings at a rate characteristic of high [*PIN*$^+$] (an average of 23.43 % of the cells had rings); 6 of 38 caused fewer cells (an average of 7.83 %) to have rings.

c= 22 of 25 [*PIN*$^+$] transfectants contained Sup35NM-GFP rings at the rate characteristic of very high [*PIN*$^+$] (an average of 39 % of cells had rings); 3 of 25 caused fewer cells (an average of 14 %) to have rings.

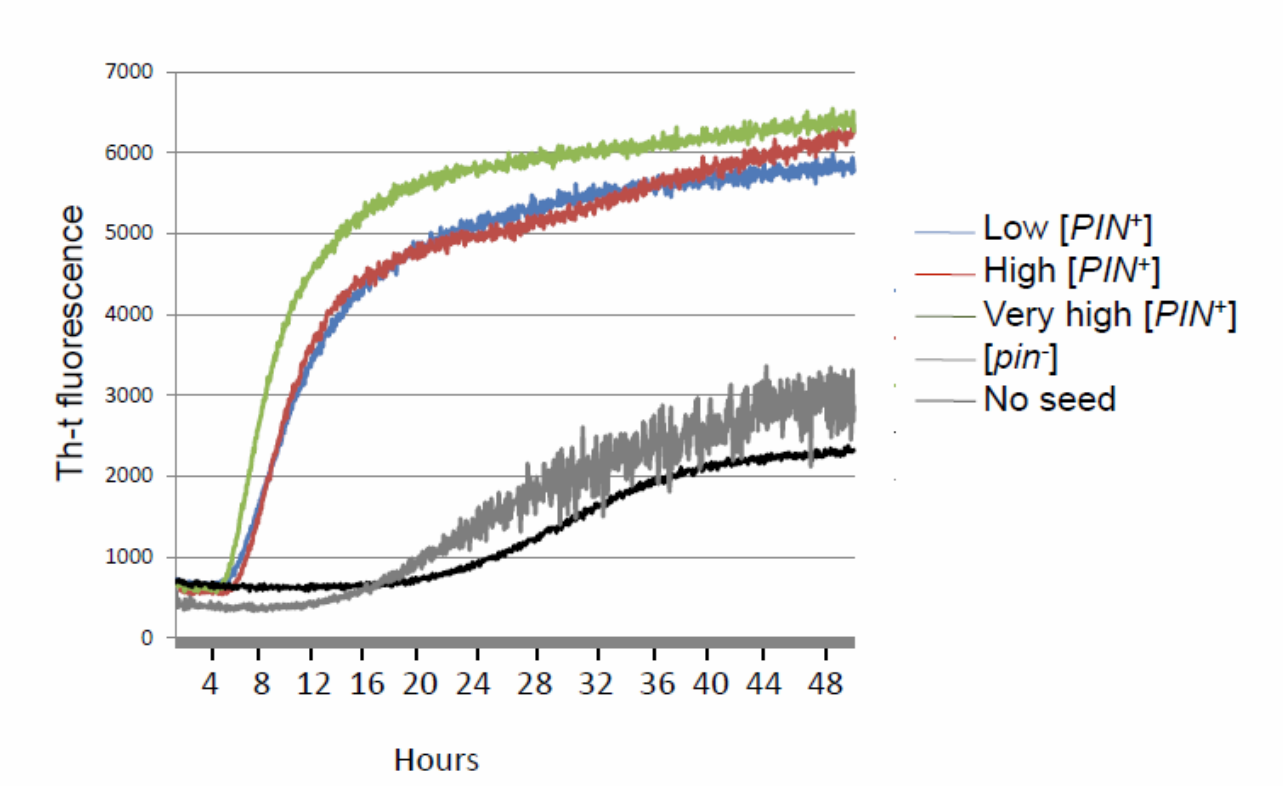


Figure 5. Purified lysate from [*PIN*$^+$] variants cross-seed variant specific [*PIN*$^+$] fibers *in vitro*. Kinetics of polymerization of 40 µM recombinant Rnq1PD (132-405) incubated with 10 sec agitation every 3 min for 48 hrs in the presence of sonicated [*PIN*$^+$] aggregates purified from low, high and very high [*PIN*$^+$] cells is shown. The rate of amyloid formation was measured by thioflavin-T dye binding which caused fluorescence. Reactions with no aggregate added (no seed) and with the "aggregates" purified from [*pin*$^-$] cells ([*pin*$^-$]) were run as controls.

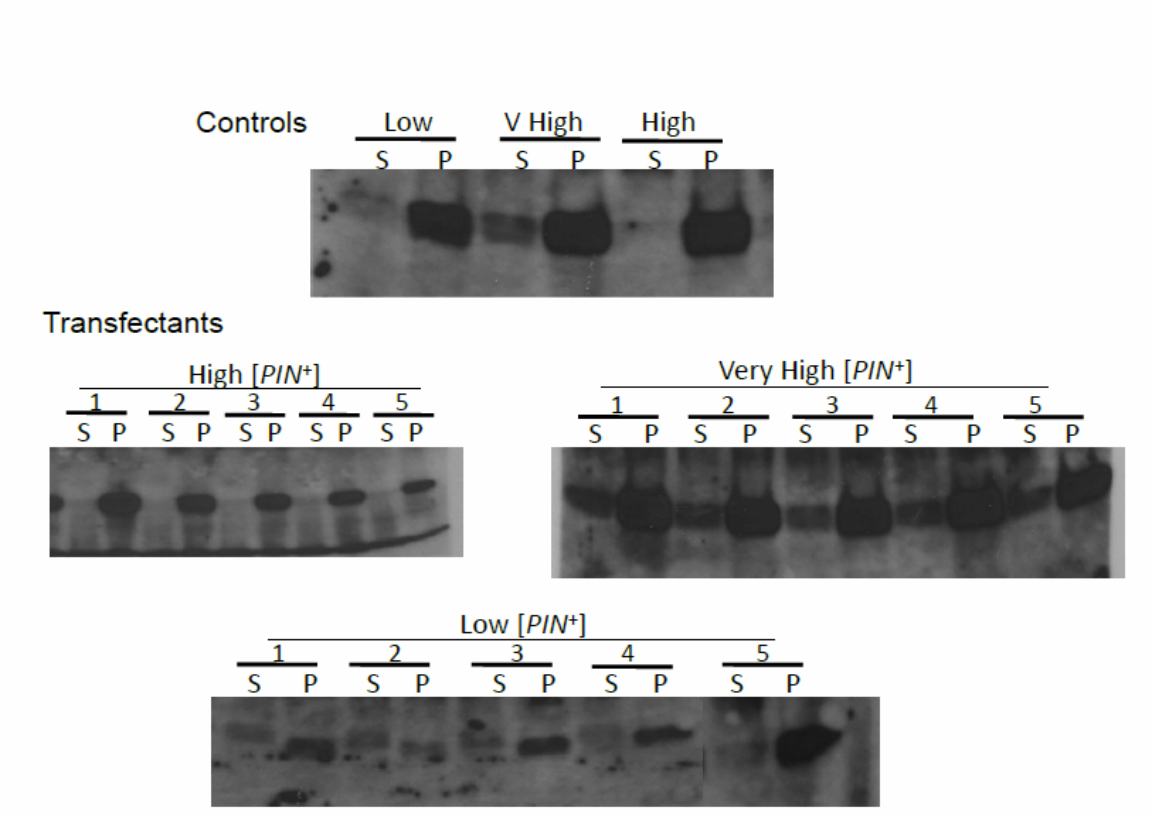


Figure 6. Determination of variant specificity of [*PIN*$^+$] transfectants by comparing the relative amounts of soluble and aggregated Rnq1 in [*PIN*$^+$] transfectants. Lysates from the [*PIN*$^+$] controls and transfectants were fractionated into soluble (S) and pelleted (P) fractions by high-speed ultracentrifugation (see Materials and Methods) and then subjected to SDS-PAGE. Western blotting used anti-Rnq1 antibody.

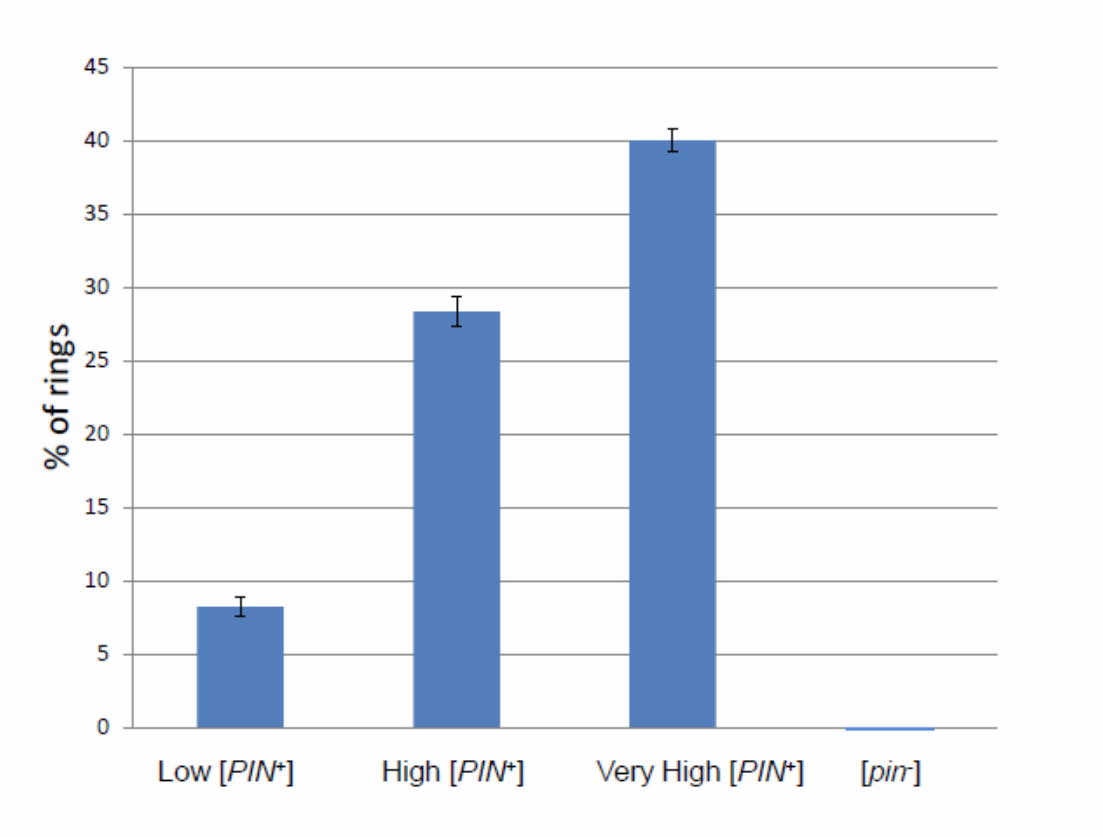


Figure 7. [*PIN*$^{+}$] variants induce different number of rings as per their [*PSI*$^{+}$] induction efficiencies. p*Cup1-SUP35NM-GFP* was transformed into low, high and very high [*PIN*$^{+}$] variants and induced with 50 µM copper sulfate overnight. After induction, the percent of cells with fluorescent rings among the total cells was scored.

did not mirror the [*PIN*$^+$] variant specific results found *in vivo* (Table 3 vs. Figure 8).

4. **[*PIN*$^+$] variants have different propagon numbers**

Propagons are the smallest infectious protein aggregates capable of transmitting a prion to progeny. Here we test the hypothesis that [*PIN*$^+$] variants with more propagons cross-seed the *de novo* appearance of [*PSI*$^+$] more efficiently.

Propagon number was estimated in different [*PIN*$^+$] variants by following the protocol developed by Cox and Tuite (Cox, Ness et al. 2003). This protocol is based on the fact that a low concentration of guanidine hydrochloride (GuHCl) blocks the generation of new prion propagons in growing cells by inhibiting the chaperone activity of Hsp104 (Ferreira, Ness et al. 2001; Jung and Masison 2001). Individual cells were grown into colonies in the presence of GuHCl where the number of cells within the colony that receive a prion propagon is an estimate of the number of propagons in the initial cell. This is because while the cells in the colony keep dividing, no new prion seeds are generated. Thus, the seeds in the initial cell segregate to daughter cells in the colony and eventually many cells do not get any seed. To determine the number of cells in each colony with a seed, the entire colony was spread on medium without GuHCl so that every cell with a single seed could make more seed and give rise to a [*PRION*$^+$] colony.

Table 3: [*PSI*$^{+}$] variants seeded *in vitro* with different [*PIN*$^{+}$] variants.

Seed	Low [*PIN*$^{+}$]		High [*PIN*$^{+}$]		Very High [*PIN*$^{+}$]	
[*PSI*$^{+}$] variant	Strong	Weak	Strong	Weak	Strong	Weak
No. of transfectants	18	10	16	20	14	18

Sup35 fibers seeded *in vitro* with the indicated [*PIN*$^{+}$] variant specific fibers were transfected into [*psi*$^{-}$][*pin*$^{-}$] yeast. Shown are the numbers of strong and weak [*PSI*$^{+}$] transfectants obtained. There were no [*PSI*$^{+}$] transfectants among 1000 transformants examined for Sup35NM fibers polymerized in the presence of mock “seed” isolated from [*pin*$^{-}$] cells. There is no statistical difference in the *in vitro* seeding of strong vs. weak [*PSI*$^{+}$] by any of the [*PIN*$^{+}$] variants.

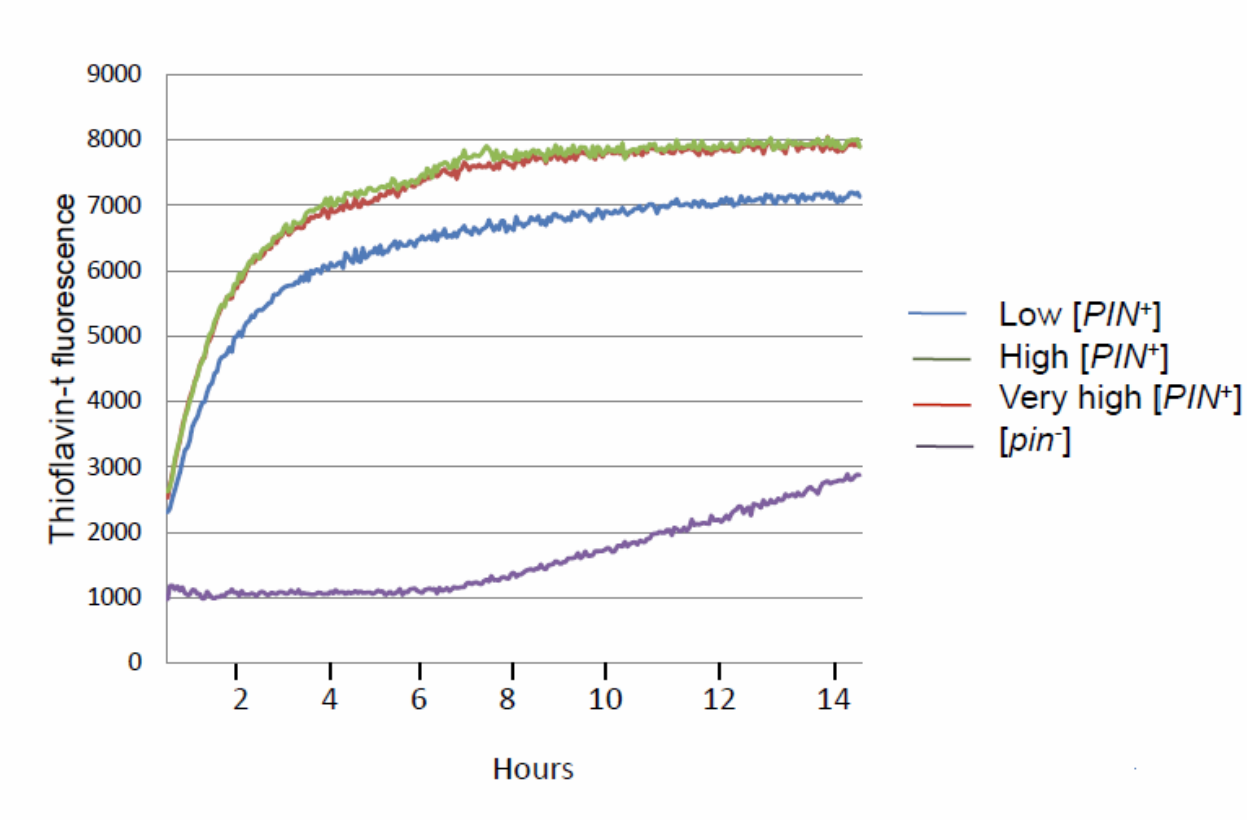


Figure 8. Kinetics of Sup35-NM polymerization in the presence of [*PIN*$^+$] fibers. Sup35NM or [*PSI*$^+$] fibers were made by incubating 40 µM purified recombinant Sup35NM for 14 hrs in the presence of 50% (v/v) variant specific sonicated [*PIN*$^+$] fibers with 10 sec agitation between every 3 min. Fluorescence caused by thioflavin-T binding to amyloid indicates fiber formation. Increasing concentrations of [*PIN*$^+$] seeds ranging from 10-50% (v/v) reduced and eventually eliminated the lag phase.

To easily score for the [*PIN*$^+$] prion we used the [*PIN*$^+$] reporter protein (RRP) strain described by Bardill *et al.* (Bardill and True 2009), where the proposed prion domain of Rnq1 (amino acids 153-405) is fused with the translation termination (MC) domain of genomic Sup35 (amino acids 124- 685). This RRP chimera aggregates in response to [*PIN*$^+$], causing nonsense suppression, which can be scored easily. We thus cytoduced low, high, and very high [*PIN*$^+$] variants into this RRP strain. The [*PIN*$^+$] variant specific characteristics of the cytoductants were confirmed with both the frequency of ring appearance and Rnq1 solubility assays.

Propagon numbers were determined in 16 individual cells (from 11 cytoductants) of each variant. Low [*PIN*$^+$] had the fewest propagons (25 ± 4.46), high [*PIN*$^+$] had the most (96 ± 17.07), and very high [*PIN*$^+$] had an intermediate number (average of 50± 5.04) (Figure 9). This finding that very high [*PIN*$^+$] variants have the intermediate level of propagons despite having the highest seeding efficiency among [*PIN*$^+$] variants shows that the propagon number alone does not determine seeding efficiency. Rather differences in the seeds' conformations may determine their cross-seeding, and hence [*PSI*$^+$] induction efficiencies.

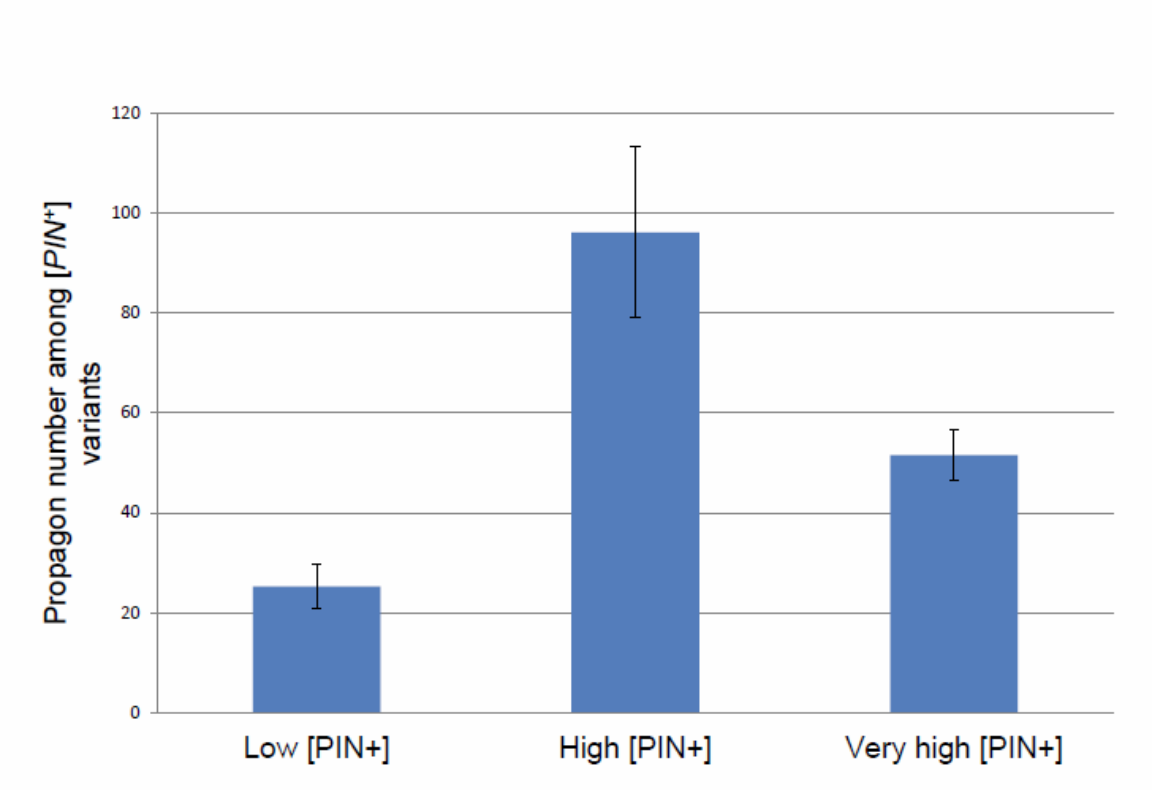


Figure 9. Low, high and very high [*PIN*$^{+}$] variants contain different characteristic numbers of [*PIN*$^{+}$] propagons. Low, high and very high [*PIN*$^{+}$] variants were cytoduced into an RRP strain (*MAT**a** ade1-14 ura3-52 leu2-3,112 trp1-289 his3-200 sup35::RMC),* where [*PIN*$^{+}$] cells are white, while [*pin*$^{-}$] cells are red (Bardill and True 2009). Individual cells without a bud were micromanipulated from log phase cultures of these cytoductants and grown into colonies for 48 hrs on medium containing 3mM GuHCl. Each microcolony was then cut out, suspended in water and plated on YPD without GuHCl. Cells were grown on YPD for 5 days. The number of non-red colonies arising from each microcolony on media with GuHCl was taken as the propagon number for the cell that grew into the microcolony.

5. [*PIN*$^+$] variants do not cause different levels of Rnq1 to be immunocaptured with Sup35 during induction of [*PSI*$^+$]

According to the cross-seeding model, during the early stages of *de novo* [*PSI*$^+$] formation [*PIN*$^+$] seeds template the aggregation of the heterologous soluble protein, Sup35. This predicts that soluble Sup35 physically interacts with the Rnq1 [*PIN*$^+$] prion seeds (Derkatch, Bradley et al. 2001; Derkatch, Uptain et al. 2004; Vitrenko, Gracheva et al. 2007; Choe, Ryu et al. 2009). Moreover, it seemed possible that seeds of very high [*PIN*$^+$] variants might bind to Sup35 more efficiently than seeds of high [*PIN*$^+$], which might bind more than seeds of low [*PIN*$^+$], thereby explaining their different efficiencies in promoting [*PSI*$^+$] induction.

We used coimmunocapture experiments to test this hypothesis by comparing the level of Rnq1 associated with Sup35 in the presence of different [*PIN*$^+$] variants. We performed coimmunocapture experiments at various time points before and after the Sup35NM-GFP forms ring aggregates indicative of *de novo* [*PSI*$^+$] induction. Low, high and very high [*PIN*$^+$] cultures transformed with p*Cup1-SUP35NM-GFP* were grown in 50 µM copper for 8, 15 and 20 hrs to induce the formation of Sup35NM-GFP rings. Sup35NM-GFP was immunocaptured with anti-GFP antibody and protein G labeled magnetic beads. Rnq1 was captured along with Sup35NM-GFP in all three [*PIN*$^+$] variants, but not in [*pin*$^-$] controls. However, the level of coimmunocaptured Rnq1 was not

reproducibly different among the three [*PIN*$^{+}$] variants (Figure 10 and 11). Nonetheless, the association of Rnq1with Sup35 in [*PIN*$^{+}$] but not [*pin*$^{-}$] cultures supports the cross-seeding model prediction that [*PIN*$^{+}$] aggregates physically interact with the heterologous Sup35 protein during induction of [*PSI*$^{+}$]. We also immunocaptured Rnq1 and attempted to determine the level of associated Sup35NM-GFP. However, the low level of Rnq1 vs. the overexpressed Sup35NM-GFP caused Sup35NM-GFP to appear even in control lanes without Rnq1 antibody.

In another approach to the question of whether the interaction of [*PIN*$^{+}$] aggregates and Sup35 is the rate-limiting step in [*PSI*$^{+}$] induction, we examined the effects of a mutation in *RNQ1* (N297S) that dramatically reduces the efficiency of [*PSI*$^{+}$] induction (Bardill and True 2009). Although the N297S mutation reduced the induction of [*PSI*$^{+}$] to less than 50% in our high [*PIN*$^{+}$] strain compared to the isogenic wild-type *RNQ1* high [*PIN*$^{+}$] strain (Figure 12), immunocapture of Sup35 in lysates of cells expressing the pRNQ::*RNQ1*-N297S mutant vs. wild-type[*PIN*$^{+}$] aggregates showed indistinguishable levels of co-isolated Rnq1 (Figure 13). This again suggests that binding of Sup35 to Rnq1 [*PIN*$^{+}$] aggregates is not the rate- limiting step in [*PSI*$^{+}$] induction.

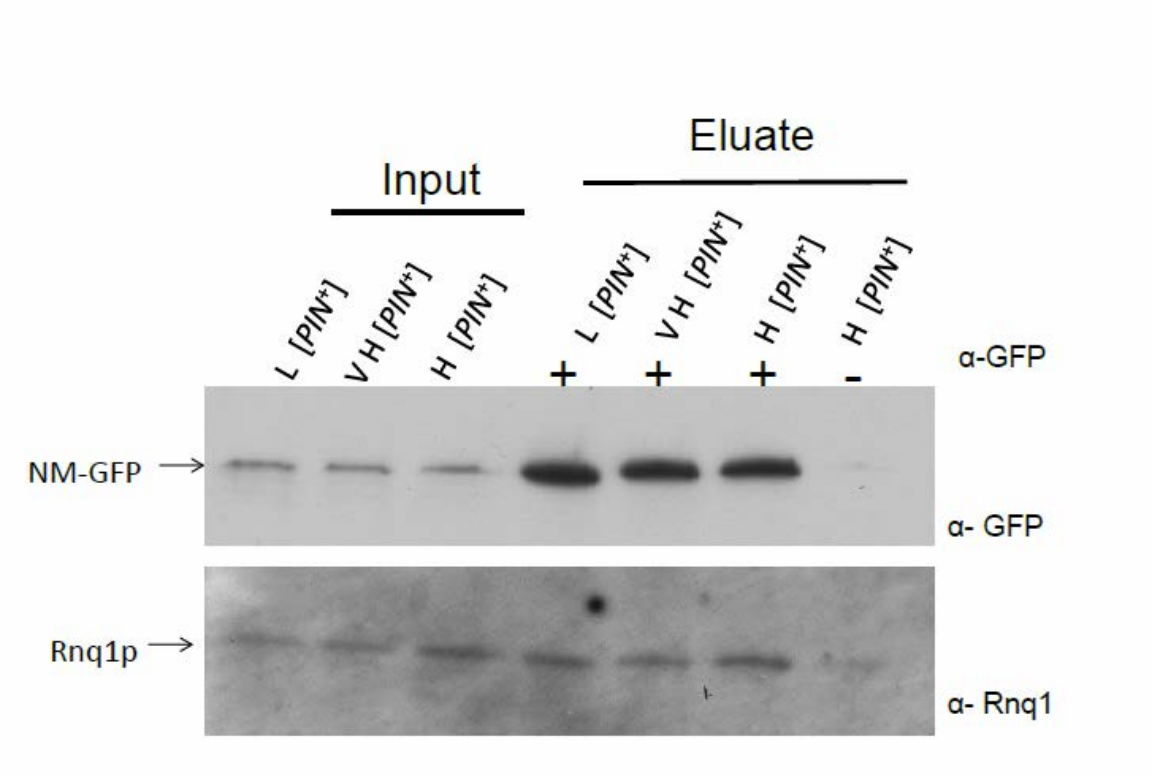
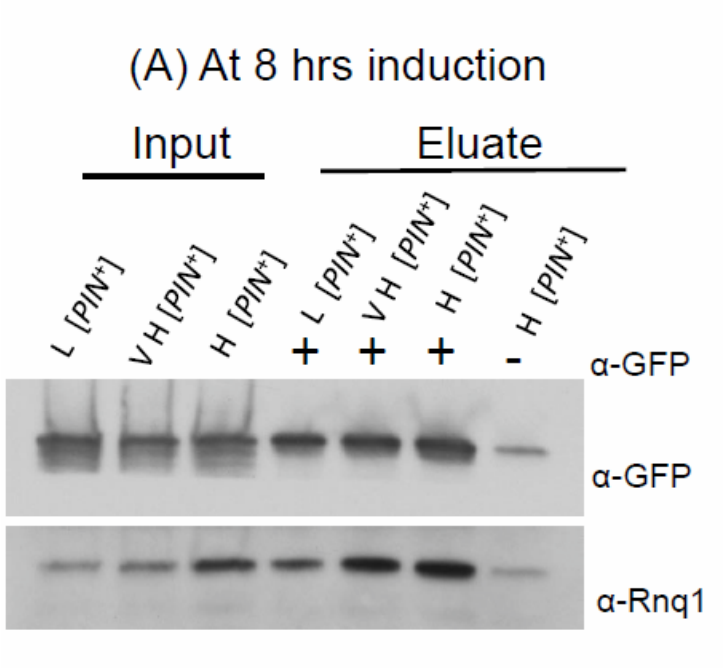


Figure 10. Rnq1 is coimmunocaptured with Sup35 during the *de novo* induction of [*PSI*$^{+}$]. The endogenous Rnq1 protein in low (L), high (H) and very high (VH) [*PIN*$^{+}$] variants are coimmunocaptured with overexpressed Sup35 with similar efficiencies. Equal amounts of lysates from cultures with Sup35NM-GFP rings that appeared following induction of p*Cup1-SUP35NM-GFP* by growth in 50 µM copper for 20 hrs, were immunocaptured on magnetic beads with (+) or without (-) anti-GFP antibody. Fractions eluted from the beads were analyzed by western blot using anti-Rnq1 antibody. Input lanes show that equal amounts of crude lysates were loaded onto the columns. Eluate lanes show the level of Rnq1 specifically coimmunocaptured with Sup35-GFP in the three [*PIN*$^{+}$] variants.


(A) At 8 hrs induction
Input
Eluate
L [PIN+]
V H [PIN+]
H [PIN+]
L [PIN+]
V H [PIN+]
H [PIN+]
H [PIN+]
+ + + -
α-GFP
α-GFP
α-Rnq1

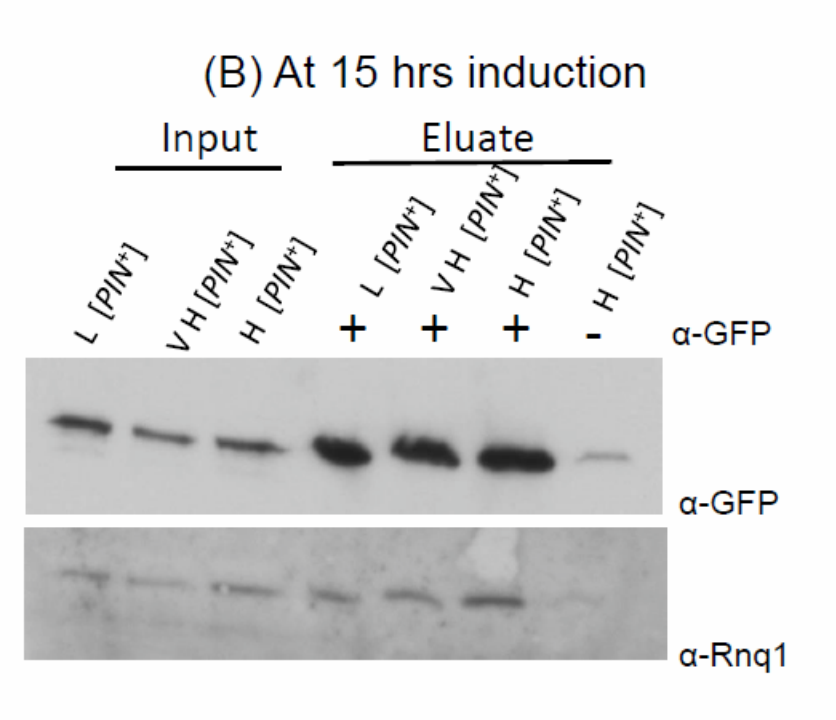

(B) At 15 hrs induction
Input
Eluate
L [PIN+]
V H [PIN+]
H [PIN+]
L [PIN+]
V H [PIN+]
H [PIN+]
H [PIN+]
+ + + -
α-GFP
α-GFP
α-Rnq1

Figure 11. [*PIN*$^{+}$] variants do not show differences in coimmuncapture of Rnq1 with Sup35 during early stages of [*PSI*$^{+}$] induction. The Rnq1 protein in low, high and very high [*PIN*$^{+}$] variants was coimmunocaptured with Sup35 with similar efficiencies. Lysates from cultures with Sup35NM-GFP following induction of p*Cup1-SUP35NM-GFP* by growth in 50 µM copper for 8 hrs (A) and 15 hrs (B) were immunocaptured with (+) or without (-) anti-GFP antibody on magnetic beads. Fractions eluted from the beads were analyzed by SDS-PAGE and western blotting using anti-Rnq1 and anti-GFP antibody.

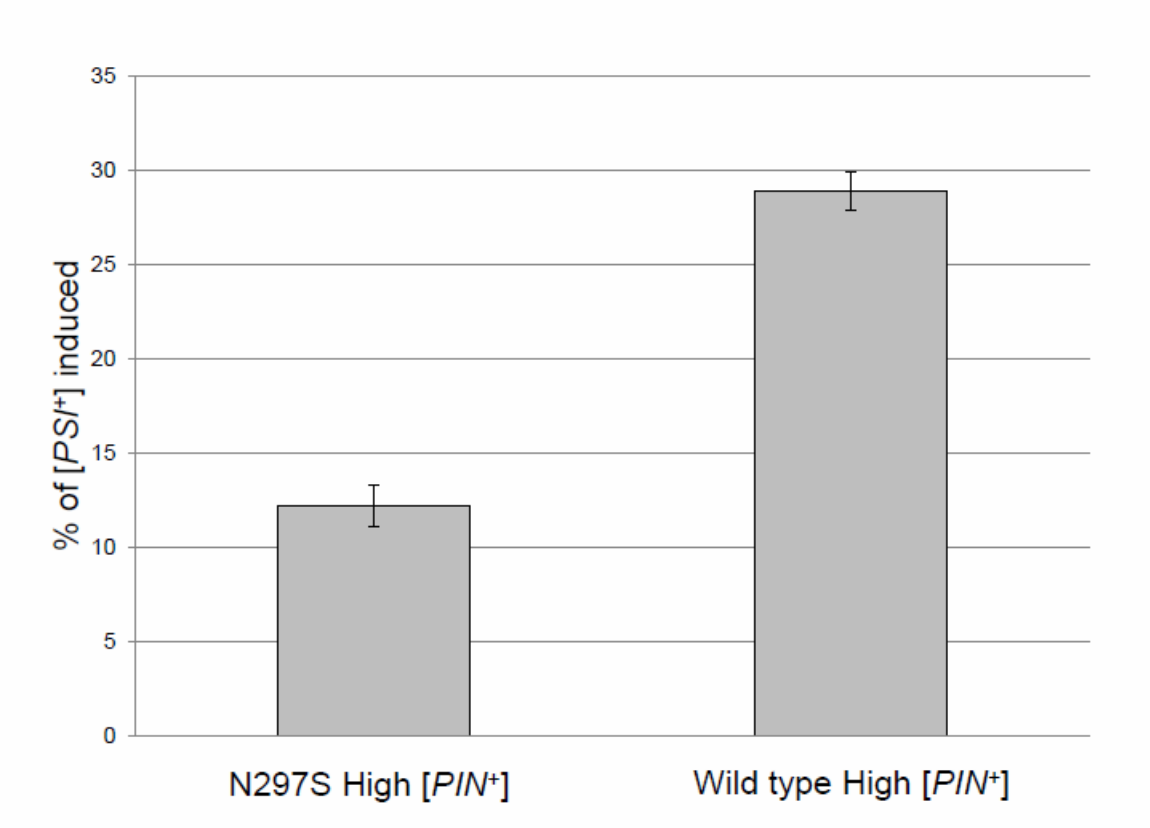


Figure 12. A [*PIN*$^+$] strain with Rnq1 mutant, N297S, induces [*PSI*$^+$] with low frequency compared to the isogenic wild type. Overexpression of Sup35NM-GFP was induced in wild-type high [*PIN*$^+$] and mutant N297S high [*PIN*$^+$] transformed with p*Cup1-SUP35NM-GFP* by overnight growth in media containing copper. After induction, ~200 cells per plate were spread on YPD and grown for 5 days. The graph shows the total number of non-red colonies counted from four transformants of both the wild-type and mutant strain.

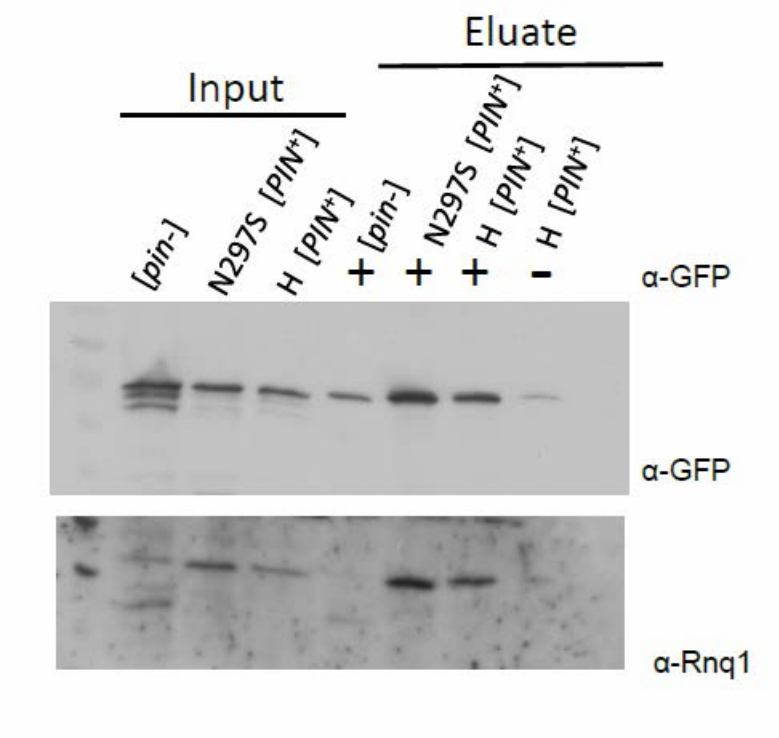


Figure 13. The Rnq1 mutant, N297S, does not alter the efficiency with which endogenous high [*PIN*$^{+}$] aggregates are coimmunocaptured with overexpressed Sup35. Sup35NM-GFP was overexpressed in high [*PIN*$^{+}$] with Rnq1 mutant N297S, high [*PIN*$^{+}$] with wild-type Rnq1and a wild-type [*pin*$^{-}$] control as in (B). Immunocapture was as in (A). Shown is a representative image of six trials. There was no reproducible difference in the efficiency of coimmunocapture of mutant vs. wild-type Rnq1 with Sup35.

6. [*PIN*$^+$] variants do not differ in the levels of associated Hsp104, Sis1 and Ssa1 chaperones

We next tested whether the variant specific [*PIN*$^+$] cross-seeding efficiencies could be caused by the binding of different levels of chaperones to [*PIN*$^+$] variant aggregates. We immunocaptured Rnq1 and compared the associated levels of the major chaperones known to participate in prion replication (Hsp104, Sis1 and Ssa1), in low, high and very high [*PIN*$^+$] variants. We did not see any reproducible differences (Figure 14).

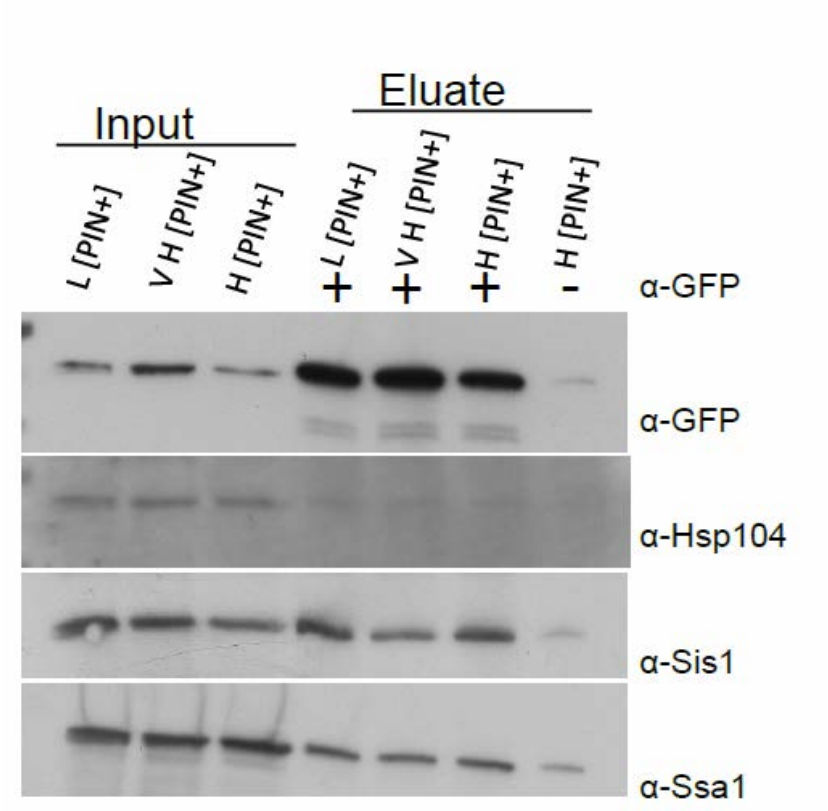


Figure 14. [*PIN*$^{+}$] variants do not show differences in the associated levels of the chaperones. [*PIN*$^{+}$] variants were transformed with the copper inducible p*Cup1-RNQ1::GFP* plasmid and induced overnight. Rnq1 was immunocaptured by magnetic beads with anti-GFP antibody. Protein was then eluted from the beads and analyzed by SDS-PAGE and western blotting.

E. Discussion

In order to explore the mechanism by which [*PIN*$^+$] promotes the *de novo* appearance of the heterologous prion [*PSI*$^+$], we examined the genetic and biochemical properties of three distinct [*PIN*$^+$] variants. We found that each variant showed statistically different preferences for promoting the appearance of strong vs. weak [*PSI*$^+$] variants. This result can be easily explained by the cross-seeding hypothesis. Indeed, since the structures of the different [*PIN*$^+$] seeds are presumably different, they would be expected to have different preferences for seeding weak and strong [*PSI*$^+$] variants that also have different structures.

However, this specificity was not reproduced *in vitro* when [*PIN*$^+$] variant Rnq1 fibers cross-seeded the *de novo* aggregation of Sup35. This could be due to slight differences between the *in vivo* and *in vitro* conditions such as distinctions between Rnq1 fiber ends created by sonication *vs*. *in vivo* fiber ends made by chaperones. Possibly, cellular factors missing *in vitro*, are directly involved in cross-seeding specificity, e.g. chaperones that affect the conformation of cross-seeded Sup35 fibers. Indeed, Hsp70s are integral components of *in vivo* [*PSI*$^+$] prion fiber assemblies (Saibil, Seybert et al. 2012). Likewise, Hsp104 enhances the ability of [*PIN*$^+$] aggregates to promote the *de novo in vivo* appearance of a heterologous prion (Kryndushkin, Engel et al. 2011).

According to the cross-seeding hypothesis one might expect conversion of heterologous soluble Sup35 protein to occur at the [*PIN*$^+$] fiber ends. In this case

the efficiency of a [*PIN*$^+$] variant in cross-seeding the appearance of [*PSI*$^+$] should depend upon the number of Rnq1 fiber ends. Indeed, *in vitro*, the efficiency with which Rnq1 fibers promoted the conversion of soluble Sup35 to amyloid fibers was dramatically increased when the number of Rnq1 fiber ends was increased by sonication (Derkatch, Uptain et al. 2004; Vitrenko, Gracheva et al. 2007).

In vivo, the number of Rnq1 fiber ends would be expected to be proportional to the number of infectious amyloid aggregates (propagons) present in the cells. Thus, we determined the number of propagons characteristic for each of the three [*PIN*$^+$] variants. While the variants differed in propagon number, this difference did not coincide with their [*PSI*$^+$] induction efficiencies. Thus, in addition to the number of available fiber ends, it is likely that the specific fiber conformations influence the efficiencies with which the different variants template the conversion of Sup35 to amyloid. This idea is also supported by our previous finding (Bradley, Edskes et al. 2002) that very high [*PIN*$^+$] contains less aggregated Rnq1 than the other [*PIN*$^+$] variants and yet promotes the appearance of [*PSI*$^+$] more efficiently than the other variants.

The coimmunocapture experiments described here support the cross-seeding hypothesis that requires overexpressed Sup35NM to physically interact with Rnq1 during [*PSI*$^+$] induction. Importantly, Rnq1 was not overexpressed in our experiments, and was not coimmunocaptured with Sup35 in control [*pin*$^-$] cells where [*PSI*$^+$] was not induced. However, we did not detect any differences

in the level of coimmunocapture of Rnq1 with overexpressed Sup35NM in cells with low, high or very high [*PIN*$^+$] variants despite their distinct differences in [*PSI*$^+$] inducing capacities. Likewise, the Rnq1mutant, N297S, which causes a 50% reduction in the efficiency with which [*PIN*$^+$] induces [*PSI*$^+$], did not cause a reduction in coimmunocapture of Rnq1 with Sup35. While transient or *in vivo* binding might not be detected by coimmunocapture, the simplest interpretation of the results is that the different [*PSI*$^+$] induction efficiencies of the [*PIN*$^+$] variants and the *RNQ1-N297S* mutant, is not caused by different levels of Rnq1 binding to Sup35. It thus appears that while the promotion of the appearance of [*PSI*$^+$] by [*PIN*$^+$] requires Sup35 to interact with Rnq1, this is not sufficient. We propose that following binding there is an additional interaction step when the actual conversion of soluble Sup35 to aggregated Sup35 occurs, and that this is the point where [*PIN*$^+$] aggregates differentially catalyze the Sup35 to [*PSI*$^+$] conversion as per their variant specificity. Similar mechanisms may explain the ability of heterologous amyloid aggregates in mammals to enhance the *de novo* appearance of amyloid associated with disease (Morales, Estrada et al. 2010; Morales, Duran-Aniotz et al. 2012).

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F. REFERENCES

Alberti, S., R. Halfmann, et al. (2009). "A systematic survey identifies prions and illuminates sequence features of prionogenic proteins." Cell **137**(1): 146-58.

Alexandrov, I. M., A. B. Vishnevskaya, et al. (2008). "Appearance and propagation of polyglutamine-based amyloids in yeast: tyrosine residues enable polymer fragmentation." J Biol Chem **283**(22): 15185-92.

Bagriantsev, S. and S. W. Liebman (2004). "Specificity of prion assembly in vivo. [PSI+] and [PIN+] form separate structures in yeast." J Biol Chem **279**(49): 51042-8.

Bagriantsev, S. N., E. O. Gracheva, et al. (2008). "Variant-specific [PSI+] infection is transmitted by Sup35 polymers within [PSI+] aggregates with heterogeneous protein composition." Mol Biol Cell **19**(6): 2433-43.

Bardill, J. P. and H. L. True (2009). "Heterologous prion interactions are altered by mutations in the prion protein Rnq1p." J Mol Biol **388**(3): 583-96.

Bessen, R. A. and R. F. Marsh (1994). "Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy." J Virol **68**(12): 7859-68.

Bradley, M. E., S. Bagriantsev, et al. (2003). "Guanidine reduces stop codon read-through caused by missense mutations in SUP35 or SUP45." Yeast **20**(7): 625-32.

Bradley, M. E., H. K. Edskes, et al. (2002). "Interactions among prions and prion "strains" in yeast." Proc Natl Acad Sci U S A **99 Suppl 4**: 16392-9.

Bradley, M. E. and S. W. Liebman (2003). "Destabilizing interactions among [PSI(+)] and [PIN(+)] yeast prion variants." Genetics **165**(4): 1675-85.

Bradley, M. E. and S. W. Liebman (2004). "The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI+] prions." Mol Microbiol **51**(6): 1649-59.

Chernoff, Y. O., I. L. Derkach, et al. (1993). "Multicopy SUP35 gene induces de-novo appearance of psi-like factors in the yeast Saccharomyces cerevisiae." Curr Genet **24**(3): 268-70.

Chernoff, Y. O., S. L. Lindquist, et al. (1995). "Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]." Science **268**(5212): 880-4.

Choe, Y. J., Y. Ryu, et al. (2009). "Increased [PSI+] appearance by fusion of Rnq1 with the prion domain of Sup35 in Saccharomyces cerevisiae." Eukaryot Cell **8**(7): 968-76.

Clos, J. and S. Brandau (1994). "pJC20 and pJC40--two high-copy-number vectors for T7 RNA polymerase-dependent expression of recombinant genes in Escherichia coli." Protein Expr Purif **5**(2): 133-7.

Cohen, F. E. and S. B. Prusiner (1998). "Pathologic conformations of prion proteins." Annu Rev Biochem **67**: 793-819.

Collinge, J., K. C. Sidle, et al. (1996). "Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD." Nature **383**(6602): 685-90.

Cox, B., F. Ness, et al. (2003). "Analysis of the generation and segregation of propagons: entities that propagate the [PSI+] prion in yeast." Genetics **165**(1): 23-33.

Cox, B. S. (1965). "[*PSI*], a cytoplasmic suppressor of super-suppressors in yeast." Heredity **20**: 505-521.

Cox, B. S., L. J. Byrne, et al. (2007). "Prion stability." Prion **1**(3): 170-8.

Cox, B. S., M. F. Tuite, et al. (1988). "The psi factor of yeast: a problem in inheritance." Yeast **4**(3): 159-78.

Derkatch, I. L., M. E. Bradley, et al. (2001). "Prions affect the appearance of other prions: the story of [PIN(+)]." Cell **106**(2): 171-82.

Derkatch, I. L., M. E. Bradley, et al. (1998). "Overexpression of the SUP45 gene encoding a Sup35p-binding protein inhibits the induction of the de novo appearance of the [PSI+] prion." Proc Natl Acad Sci U S A **95**(5): 2400-5.

Derkatch, I. L., M. E. Bradley, et al. (2000). "Dependence and independence of [PSI(+)] and [PIN(+)]: a two-prion system in yeast?" EMBO J **19**(9): 1942-52.

Derkatch, I. L., M. E. Bradley, et al. (1997). "Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae." Genetics **147**(2): 507-19.

Derkatch, I. L., Y. O. Chernoff, et al. (1996). "Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae." Genetics **144**(4): 1375-86.

Derkatch, I. L., S. M. Uptain, et al. (2004). "Effects of Q/N-rich, polyQ, and non-polyQ amyloids on the de novo formation of the [PSI+] prion in yeast and aggregation of Sup35 in vitro." Proc Natl Acad Sci U S A **101**(35): 12934-9.

DiSalvo, S., A. Derdowski, et al. (2011). "Dominant prion mutants induce curing through pathways that promote chaperone-mediated disaggregation." Nat Struct Mol Biol **18**(4): 486-92.

Du, Z., K.-W. Park, et al. (2008). "Newly identified prion linked to the chromatin-remodeling factor Swi1 in Saccharomyces cerevisiae." Nat Genet **40**(4): 460-465.

Ferreira, P. C., F. Ness, et al. (2001). "The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation." Mol Microbiol **40**(6): 1357-69.

Fraser, H. and A. G. Dickinson (1973). "Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation." J Comp Pathol **83**(1): 29-40.

Gotz, J., F. Chen, et al. (2001). "Formation of neurofibrillary tangles in P301l tau transgenic mice induced by Abeta 42 fibrils." Science **293**(5534): 1491-5.

Higurashi, T., J. K. Hines, et al. (2008). "Specificity of the J-protein Sis1 in the propagation of 3 yeast prions." Proc Natl Acad Sci U S A **105**(43): 16596-601.

Hosoda, N., T. Kobayashi, et al. (2003). "Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation." J Biol Chem **278**(40): 38287-91.

Jung, G. and D. C. Masison (2001). "Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions." Curr Microbiol **43**(1): 7-10.

Kadnar, M. L., G. Articov, et al. (2010). "Distinct type of transmission barrier revealed by study of multiple prion determinants of Rnq1." PLoS Genet **6**(1): e1000824.

Kalastavadi, T. and H. L. True (2010). "Analysis of the [RNQ+] prion reveals stability of amyloid fibers as the key determinant of yeast prion variant propagation." J Biol Chem **285**(27): 20748-55.

King, C. Y. (2001). "Supporting the structural basis of prion strains: induction and identification of [PSI] variants." J Mol Biol **307**(5): 1247-60.

King, C. Y., H. L. Wang, et al. (2006). "Transformation of yeast by infectious prion particles." Methods **39**(1): 68-71.

Kochneva-Pervukhova, N. V., M. B. Chechenova, et al. (2001). "[Psi(+)] prion generation in yeast: characterization of the 'strain' difference." Yeast **18**(6): 489-97.

Krishnan, R. and S. L. Lindquist (2005). "Structural insights into a yeast prion illuminate nucleation and strain diversity." Nature **435**(7043): 765-72.

Kryndushkin, D. S., I. M. Alexandrov, et al. (2003). "Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104." J Biol Chem **278**(49): 49636-43.

Kryndushkin, D. S., A. Engel, et al. (2011). "Molecular chaperone Hsp104 can promote yeast prion generation." Genetics **188**(2): 339-48.

Lasagna-Reeves, C. A., D. L. Castillo-Carranza, et al. (2010). "Preparation and characterization of neurotoxic tau oligomers." Biochemistry **49**(47): 10039-41.

Liebman, S. W. and Y. O. Chernoff (2012). "Prions in yeast." Genetics **191**(4): 1041-72.

Lin, J. Y., T. Y. Liao, et al. (2011). "Inter-allelic prion propagation reveals conformational relationships among a multitude of [PSI] strains." PLoS Genet **7**(9): e1002297.

Liu, J. J., N. Sondheimer, et al. (2002). "Changes in the middle region of Sup35 profoundly alter the nature of epigenetic inheritance for the yeast prion [PSI+]." Proc Natl Acad Sci U S A **99 Suppl 4**: 16446-53.

Masison, D. C. and R. B. Wickner (1995). "Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells." Science **270**(5233): 93-5.

Morales, R., C. Duran-Aniotz, et al. (2012). "De novo induction of amyloid-beta deposition in vivo." Mol Psychiatry **17**(12): 1347-53.

Morales, R., L. D. Estrada, et al. (2010). "Molecular cross talk between misfolded proteins in animal models of Alzheimer's and prion diseases." J Neurosci **30**(13): 4528-35.

Moriyama, H., H. K. Edskes, et al. (2000). "[URE3] prion propagation in Saccharomyces cerevisiae: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p." Mol Cell Biol **20**(23): 8916-22.

O'Nuallain, B., A. D. Williams, et al. (2004). "Seeding specificity in amyloid growth induced by heterologous fibrils." J Biol Chem **279**(17): 17490-9.

Oesch, B., D. Westaway, et al. (1985). "A cellular gene encodes scrapie PrP 27-30 protein." Cell **40**(4): 735-46.

Osherovich, L. Z. and J. S. Weissman (2001). "Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion." Cell **106**(2): 183-94.

Pan, K. M., M. Baldwin, et al. (1993). "Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins." Proceedings of the National Academy of Sciences of the United States of America **90**(23): 10962-10966.

Patel, B. K., J. Gavin-Smyth, et al. (2009). "The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion." Nat Cell Biol **11**(3): 344-9.

Patel, B. K. and S. W. Liebman (2007). ""Prion-proof" for [PIN+]: infection with in vitro-made amyloid aggregates of Rnq1p-(132-405) induces [PIN+]." J Mol Biol **365**(3): 773-82.

Prusiner, S. B. (1998). "Prions." Proc Natl Acad Sci U S A **95**(23): 13363-83.

Saibil, H. R., A. Seybert, et al. (2012). "Heritable yeast prions have a highly organized three-dimensional architecture with interfiber structures." Proc Natl Acad Sci U S A **109**(37): 14906-11.

Salnikova, A. B., D. S. Kryndushkin, et al. (2005). "Nonsense suppression in yeast cells overproducing Sup35 (eRF3) is caused by its non-heritable amyloids." J Biol Chem **280**(10): 8808-12.

Schlumpberger, M., S. B. Prusiner, et al. (2001). "Induction of distinct [URE3] yeast prion strains." Mol Cell Biol **21**(20): 7035-46.

Serio, T. R., A. G. Cashikar, et al. (2000). "Nucleated conformational conversion and the replication of conformational information by a prion determinant." Science **289**(5483): 1317-21.

Serio, T. R., A. G. Cashikar, et al. (1999). "Yeast prion [psi +] and its determinant, Sup35p." Methods Enzymol **309**: 649-73.

Sharma, J. and S. W. Liebman (2012). "[PSI(+)] prion variant establishment in yeast." Mol Microbiol.

Sherman, F., Fink, G. R. & Hicks, J. B. (1986). *Methods in Yeast Genetics*. Plainview, New York, Cold Spring Harbor Lab.

Shorter, J. and S. Lindquist (2004). "Hsp104 catalyzes formation and elimination of self-replicating Sup35 prion conformers." Science **304**(5678): 1793-7.

Sondheimer, N. and S. Lindquist (2000). "Rnq1: an epigenetic modifier of protein function in yeast." Mol Cell **5**(1): 163-72.

Soto, C. (2003). "Unfolding the role of protein misfolding in neurodegenerative diseases." Nat Rev Neurosci **4**(1): 49-60.

Stansfield, I., K. M. Jones, et al. (1995). "The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in Saccharomyces cerevisiae." Embo J **14**(17): 4365-73.

Tanaka, M., S. R. Collins, et al. (2006). "The physical basis of how prion conformations determine strain phenotypes." Nature **442/3**: 585-589.

Tanaka, M., S. R. Collins, et al. (2006). "The physical basis of how prion conformations determine strain phenotypes." Nature **442**(7102): 585-9.

Tanaka, M. and J. S. Weissman (2006). "An efficient protein transformation protocol for introducing prions into yeast." Methods Enzymol **412**: 185-200.

Ter-Avanesyan, M. D., A. R. Dagkesamanskaya, et al. (1994). "The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast Saccharomyces cerevisiae." Genetics **137**(3): 671-6.

Ter-Avanesyan, M. D., V. V. Kushnirov, et al. (1993). "Deletion analysis of the SUP35 gene of the yeast Saccharomyces cerevisiae reveals two non-overlapping functional regions in the encoded protein." Mol Microbiol **7**(5): 683-92.

Tipton, K. A., K. J. Verges, et al. (2008). "In vivo monitoring of the prion replication cycle reveals a critical role for Sis1 in delivering substrates to Hsp104." Mol Cell **32**(4): 584-91.

Toyama, B. H., M. J. Kelly, et al. (2007). "The structural basis of yeast prion strain variants." Nature **449**(7159): 233-7.

Toyama, B. H. and J. S. Weissman (2011). "Amyloid structure: conformational diversity and consequences." Annu Rev Biochem **80**: 557-85.

Tuite, M. F., R. Marchante, et al. (2011). "Fungal prions: structure, function and propagation." Top Curr Chem **305**: 257-98.

Tuite, M. F., C. R. Mundy, et al. (1981). "Agents that cause a high frequency of genetic change from [psi+] to [psi-] in Saccharomyces cerevisiae." Genetics **98**(4): 691-711.

Tuite, M. F. and T. R. Serio (2010). "The prion hypothesis: from biological anomaly to basic regulatory mechanism." Nat Rev Mol Cell Biol **11**(12): 823-33.

Uptain, S. M., G. J. Sawicki, et al. (2001). "Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion." Embo J **20**(22): 6236-45.

Verges, K. J., M. H. Smith, et al. (2011). "Strain conformation, primary structure and the propagation of the yeast prion [PSI+]." Nat Struct Mol Biol **18**(4): 493-9.

Vishveshwara, N., M. E. Bradley, et al. (2009). "Sequestration of essential proteins causes prion associated toxicity in yeast." Mol Microbiol **73**(6): 1101-14.

Vitrenko, Y. A., E. O. Gracheva, et al. (2007). "Visualization of aggregation of the Rnq1 prion domain and cross-seeding interactions with Sup35NM." J Biol Chem **282**(3): 1779-87.

Vitrenko, Y. A., M. E. Pavon, et al. (2007). "Propagation of the [PIN+] prion by fragments of Rnq1 fused to GFP." Curr Genet **51**(5): 309-19.

Wickner, R. B. (1994). "[URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae." Science **264**(5158): 566-9.

Wickner, R. B., H. K. Edskes, et al. (2012). "Prion diseases of yeast: amyloid structure and biology." Semin Cell Dev Biol **22**(5): 469-75.

Wickner, R. B., Liebman, S.W. and Saupe, S.J. (2004). Chapter 7 "Prions of Yeast and Filamentous Fungi: [URE3^{+}], [PSI^{+}], [PIN^{+}], and [Het-s]", , Cold Spring Harbor Laboratory Press.

Wickner, R. B., D. C. Masison, et al. (1995). "[PSI] and [URE3] as yeast prions." Yeast **11**(16): 1671-85.

Zhou, P., I. L. Derkatch, et al. (2001). "The relationship between visible intracellular aggregates that appear after overexpression of Sup35 and the yeast prion-like elements [PSI(+)] and [PIN(+)]." Mol Microbiol **39**(1): 37-46.

Zhou, P., I. L. Derkatch, et al. (1999). "The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3." EMBO J **18**(5): 1182-91.

CHAPTER IV

CONCLUSIONS

IV. CONCLUSIONS

The aggregated form of the prion protein,PrP^{Sc}, deposits in the brain tissues leading to toxicity with the initial cause being either the infectious inoculum or more often, the spontaneous generation of prion aggregates. With the recent advances in the field similar mechanism has been proposed for other more common neurodegenerative diseases which were earlier not thought to be infectious (Holmes and Diamond, 2012), (Prusiner, 1998). Amyloid and prion disease share the common feature of aggregation leading to toxicity except that one is highly transmissible among humans and across species (Aguzzi and O'Connor, 2010).

Although, the question of whether yeast prions provide evolutionary advantage or are detrimental to their host cells remains unanswered, studies of yeast prions have advanced our understanding of prion biology to a great extent. They have been a very useful model to study prion birth, propagation, variant phenomenon and toxicity.

A. Establishment of [*PSI*$^{+}$] variants

A very important question of prion biology remains unsolved- how a single protein-only infectious agent can encode the information required to specify distinct disease phenotypes (in mammals) or heritable traits (in yeast). Similar to

the mammalian prion, PrP^{Sc}, individual yeast prions have been shown to exist in multiple conformations that manifest distinct phenotypes (Derkatch *et al.*, 1996). [*PSI*$^{+}$] variants of different phenotypes were obtained when the Sup35 prion domain was overexpressed in the same strain. It was not known if those multiple conformers arose in different cells or if more than one conformer can be produced in the same cell. The prevailing hypothesis was that once one kind of [*PSI*$^{+}$] aggregate was produced in a cell it would efficiently attract all soluble molecules of that protein and convert them to the aggregate's conformation, precluding the formation of aggregate with different conformations. It was thought that the multiple variants that arose in one culture originated from different cells. On testing this hypothesis, we discovered that more than one kind of prion conformers can be formed in a single cell. In chapter 2, we showed, when Sup35-GFP was overexpressed until the cell formed a ring or line like aggregate indicative of *de novo* [*PSI*$^{+}$] induction and examined, 40 % of the viable ring cells gave rise to progeny of more than one variant type. Since overexpressing Sup35NM-GFP in established strong or weak [*PSI*$^{+}$] variant, did not alter the variant type, it appeared that more than one kind of conformers could be generated in a single cell during the *de novo* induction of [*PSI*$^{+}$]. Pedigree analysis showed that progeny from mothers containing more than one variant, eventually give rise to some cells with only one kind of variant.

We also discovered, a unique kind of variant, unspecified [*PSI*$^{+}$], which always gave rise to strong, weak and unspecified [*PSI*$^{+}$] daughters along with [*psi*-]. It was previously thought that only newly appearing [*PSI*$^{+}$] is unstable and this instability caused the loss of the prion and not to its conversion to another variant (Bradley and Liebman, 2004; Derkatch *et al.*, 1996). In contrast, this unspecified variant continued to segregate into more than one kind of variant indefinitely (checked for 25 passes). Surprisingly, ~5% of newly induced [*PSI*$^{+}$] were of the unspecified type.

Despite giving rise to progeny of more than one kind of variant, unspecified [*PSI*$^{+}$] cells only consisted of unspecified propagons. This suggested that unspecified propagons could be the primitive state of propagons prior to their maturation into a specified strong or weak variant.propagon. We propose that after the birth of every prion propagon, it goes through an adaptation or maturation phase. This phenomenon is analogous to the proposed mammalian prion adaptation process where the primary passage is associated with prolonged incubation periods whereas subsequent passages are much shorter with higher lethality rate in the same type of host (Hill *et al.*, 2000).

B. Variant specific differences of [*PIN*$^{+}$] affect the appearance of [*PSI*$^{+}$]

The preexisting prion [*PIN*$^{+}$] is associated with the enhanced appearance of [*PSI*$^{+}$] following the overexpression of the prion protein Sup35 or Sup35-NM

(Derkatch *et al.*, 1997). The exact mechanism behind this heterologous prion effect remains unclear, but considerable evidences supports a cross-seeding hypothesis (Derkatch *et al.*, 2001). According to this hypothesis, [*PIN*$^{+}$] acts as an inefficient template to cross-seed Sup35 during [*PSI*$^{+}$] formation. Depending upon the variant type of the [*PIN*$^{+}$] prion, the frequency of appearance of [*PSI*$^{+}$] varies. In chapter 3, we tried to explore the mechanism of differential rates of [*PSI*$^{+}$] induction among the [*PIN*$^{+}$] variants. First, we investigated if there was any specificity between the variant type of [*PIN*$^{+}$] seed and the [*PSI*$^{+}$] variant type induced. If there were, it would support the hypothesis that the [*PIN*$^{+}$] seeds directly interact with Sup35 and transfer variant specific character when seeding the aggregation of Sup35 into [*PIN*$^{+}$]. We found that the low [*PIN*$^{+}$] variant prefered to induce weak [*PSI*$^{+}$] whereas very high [*PIN*$^{+}$] had a preference to induce strong [*PSI*$^{+}$]. When we addressed the same question through an *in vitro* approach by using variant specific Rnq1 fibers as seed to make [*PSI*$^{+}$] fibers, the preferences could not established. This suggested a role for cellular factors and chaperones during cross-seeding.

Although a role for chaperones in transmission of mammalian prions has not established, chaperones have been shown to be integral part of yeast prion propagation and maintenance. Studies have shown chaperones (eg. Hsp104 with the aid of Hsp70 and Hsp40) shear prion aggregates into prion seeds that are transmitted to progeny cells. We propose that chaperones or cellular factors

either reshaping or remodeling Rnq1 seeds before cross-seeding or they assist in acquiring appropriate conformation of Sup35 molecules following cross-seeding. Evidences from recent studies suggest that overexpression of Hsp104 enhances the cross-seeding ability of [*PIN*$^+$] for the appearance of heterologous prion (Kryndushkin *et al.*, 2011). Additionally Hsp104 was shown to promote amyloid fibrillization of prion proteins *in vitro* suggesting it might help in initial nucleation event (Krzewska and Melki, 2006; Shorter and Lindquist, 2006).

C. Future directions

Our work investigating the role of [*PIN*$^+$] in the appearance of [*PSI*$^+$] and establishment of [*PSI*$^+$] variants brings up a number of new questions. Of considerable interest is the identification of the cellular factors or chaperones involved in the creating the preference specificity seen *in vivo* in [*PIN*$^+$]-Sup35 cross-seeding step. One approach to this question could be asking if the variant specificity of [*PSI*$^+$] induction gets altered if certain chaperones are mutated or depleted *in vivo*? Once these factors are identified, one could focus on unraveling the molecular basis of their effects on the Sup35-Rnq1 interaction. Key to these questions will be the molecular establishment of the structure of [*PIN*$^+$] aggregates of the different variants.

D. REFERENCES

Aguzzi, A., and O'Connor, T. (2010) Protein aggregation diseases: pathogenicity and therapeutic perspectives. *Nat Rev Drug Discov* 9: 237-248.

Bradley, M.E., and Liebman, S.W. (2004) The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI+] prions. *Mol Microbiol* 51: 1649-1659.

Derkatch, I.L., Chernoff, Y.O., Kushnirov, V.V., Inge-Vechtomov, S.G., and Liebman, S.W. (1996) Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae. *Genetics* 144: 1375-1386.

Derkatch, I.L., Bradley, M.E., Zhou, P., Chernoff, Y.O., and Liebman, S.W. (1997) Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae. *Genetics* 147: 507-519.

Derkatch, I.L., Bradley, M.E., Hong, J.Y., and Liebman, S.W. (2001) Prions affect the appearance of other prions: the story of [PIN(+)]. *Cell* 106: 171-182.

Hill, A.F., Joiner, S., Linehan, J., Desbruslais, M., Lantos, P.L., and Collinge, J. (2000) Species-barrier-independent prion replication in apparently resistant species. *Proc Natl Acad Sci U S A* 97: 10248-10253.

Holmes, B.B., and Diamond, M.I. (2012) Cellular mechanisms of protein aggregate propagation. *Curr Opin Neurol* 25: 721-726.

Kryndushkin, D.S., Engel, A., Edskes, H., and Wickner, R.B. (2011) Molecular chaperone Hsp104 can promote yeast prion generation. *Genetics* 188: 339-348.

Krzewska, J., and Melki, R. (2006) Molecular chaperones and the assembly of the prion Sup35p, an in vitro study. *Embo J* 25: 822-833.

Prusiner, S.B. (1998) Prions. *Proc Natl Acad Sci U S A* 95: 13363-13383.

Shorter, J., and Lindquist, S. (2006) Destruction or potentiation of different prions catalyzed by similar Hsp104 remodeling activities. *Mol Cell* 23: 425-438.

APPENDIX

TEMPERATURE HAS EFFECT ON THE *DE NOVO* APPEARANCE OF [*PSI*$^{+}$] VARIANT *IN VIVO*

INTRODUCTION

Translational termination factor, Sup35, when misfolded acquires multiple conformations of [*PSI*$^{+}$]. Those aggregated forms of Sup35 can give rise to various phenotypes of [*PSI*$^{+}$]. Inactivation of Sup35 causes suppression of the nonsense mutation in the strains carrying *ade 1-14* reporter gene. [*psi-*] colonies are red as they accumulate the intermediate product of Ade1 biosynthesis. [*PSI*$^{+}$] colonies vary in the degree of nonsense suppression and hence exhibit range of non-red color depending on degree of Sup35 inactivation. Thus, these non-red colored colonies are actually representative phenotypes of various [*PSI*$^{+}$] variants.

[*PSI*$^{+}$] variants differ in mitotic stability (Derkatch *et al.*, 1996), in interaction with cellular chaperones (Kushnirov *et al.*, 2000), in solubility and functionality of the Sup35 protein (Derkatch *et al.*, 1996; Kochneva-Pervukhova *et al.*, 2001; Uptain *et al.*, 2001; Zhou *et al.*, 1999). They also differ in their ability to recruit Sup35 mutants (King, 2001). Sup35 is a modular protein and [*PSI*$^{+}$] prion activity is localized at asparagines/glutamine (Q/N) rich domain which is separable from the translation termination domain. In *in vitro* conditions, recombinant purified protein containing prion domain Sup35-NM can spontaneously form amyloid fibers and can adopt several form of fibrillar structure. It was reported that different amyloid forms of Sup35-NM could be generated by spontaneously polymerizing Sup35-NM at 4ºC or 37ºC and were

called as Sc4 and Sc37 respectively (Krishnan and Lindquist, 2005; Tanaka *et al.*, 2004; Tanaka *et al.*, 2005). On transfecting these Sc4 and Sc37 amyloids into [*psi*-] yeasts, it lead to readily distinguishable prion variants specific to the temperature. Sc4 amyloid gave rise to strong and Sc37 gave rise to weak variant of [*PSI*$^{+}$] (Tanaka *et al.*, 2006a). On investigating the structure of these Sc37 and Sc4 amyloid, Sc37 was shown to contain expanded amyloid core compared to Sc4 amyloid (Krishnan and Lindquist, 2005; Toyama *et al.*, 2007). Hence, extended structure in Sc37, increased fiber stability and decreased the prion fragmentation by chaperones giving rise to weaker variants (Tanaka *et al.*, 2006b). In contrast smaller amyloid core of Sc4 made them more breakable, and are broken down into smaller fibers which more actively recruit soluble Sup35 and give rise to strong [*PSI*$^{+}$] phenotype (Toyama and Weissman, 2011).

Here, we studied if temperature has similar variant specific effect in the population of [*PSI*$^{+}$] arising following *de novo* induction.

METHODS

Yeast color assay

The yeast strain used in this study (*MAT**a** ade1-14 ura3-52 leu2-3, 112 trp1-289 his3-200* [*psi*$^{-}$] [*PIN*$^{+}$]High [*PIN*$^{+}$]) (Chernoff *et al.*, 1995) has mutant *ade1-14* allele. It has a premature stop codon, hence [*psi*-] cells containing

functional Sup35 will recognize and cannot synthesize full length Ade1 protein. They are red colored in the rich medium like YPD . In [*PSI*$^{+}$] cells, majority of Sup35 is in aggregated form as a result occasionally such premature stop codons get read through and full length Ade1 protein is synthesized and yeast cells display white color on YPD.

The plasmid p*Cup1:SUP35NM-GFP* (-Leu) was used for the induction of [*PSI*$^{+}$] (Zhou *et al.*, 2001).

[*PSI*$^{+}$] induction

L1749 strain was transformed with p*Cup1:SUP35NM-GFP,* and three transformants were selected on –Leu for induction. Induction was done by replica plating those 3 transformants on 4 plates of –leu medium containing 50 µM copper and incubated at room temperature (RT), 30ºC, 37ºC and 4ºC each. Incubation at 4ºC was carried out for one week whereas for other temperatures incubation was done for 48 hrs. After induction, cells were suspended in water and plated on YPD plates (200 cells/plate). After 5 days incubation at 30ºC, the plates were examined and non-red colonies counted.

Results and discussion

Here we asked if temperature during induction has any effect in the types [*PSI*$^{+}$] variants arising *de novo*. We overexpressed Sup35NM-GFP at various

temperature and analyzed if certain variants were more likely to be induced at certain temperature. On scoring the [*PSI*$^{+}$] variants induced, we saw weak [*PSI*$^{+}$] variants were more likely to be induced at RT, 30ºC and 37ºC , whereas strong [*PSI*$^{+}$] variants were predominant among the population of [*PSI*$^{+}$] induced at 4ºC (Fig.1). Sectored colonies were subcloned and variant type confirmed.

This suggested, population of [*PSI*$^{+}$] variant could vary with the incubation temperature during induction. Our data is in accordance with previously made observation that strong variant fibers were formed at 4ºC and weak variant fibers were formed at 37ºC (Tanaka *et al.*, 2006a). Hence, temperature has profound effect in making the amyloid core of [*PSI*$^{+}$] fibers both in *in vivo* and *in vitro*.

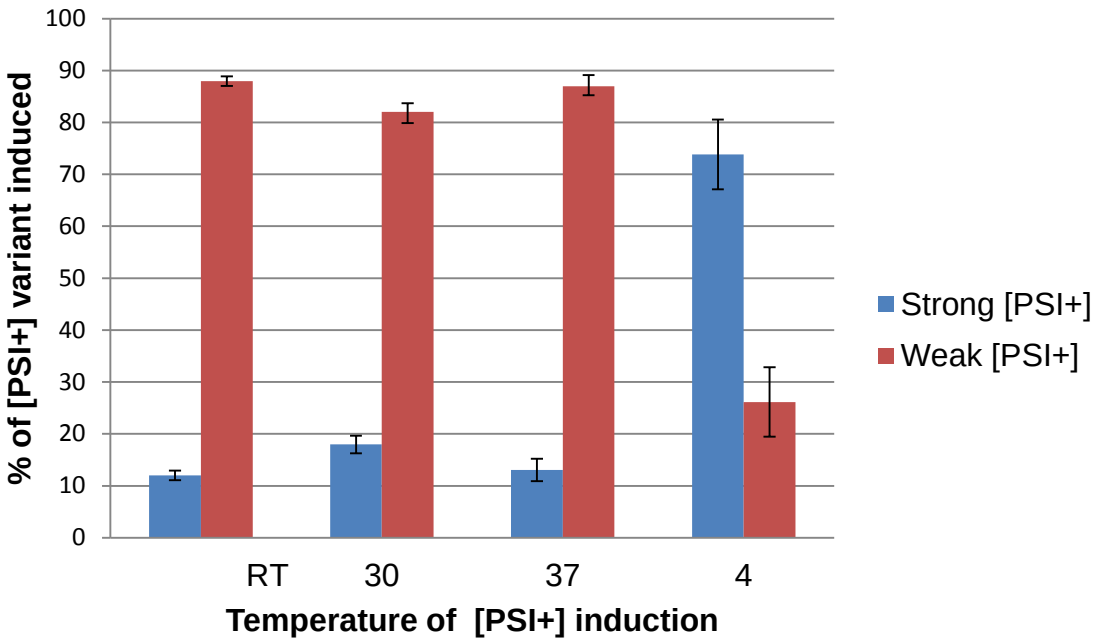

Figure 1.Temperature has effect on the variant population of [*PSI*$^{+}$] arising following *de novo* induction. L1749 strain transformed with the plasmid Sup35NM-GFP was induced in the presence of 50 µM copper at different temperature. Induction was done for 48 hrs at RT, 30ºC and 37º C whereas cells incubated at 4ºC were induced for a week. The induced cells were suspended in water and plated on YPD plates. After incubation for 5 days at 30º C, non-red colonies were scored and tabulated. For the sectored colonies they were subcloned and variant type conformed.

REFERENCES

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G., and Liebman, S.W. (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. *Science* **268**: 880-884.

Derkatch, I.L., Chernoff, Y.O., Kushnirov, V.V., Inge-Vechtomov, S.G., and Liebman, S.W. (1996) Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae. *Genetics* **144**: 1375-1386.

King, C.Y. (2001) Supporting the structural basis of prion strains: induction and identification of [PSI] variants. *J Mol Biol* **307**: 1247-1260.

Kochneva-Pervukhova, N.V., Chechenova, M.B., Valouev, I.A., Kushnirov, V.V., Smirnov, V.N., and Ter-Avanesyan, M.D. (2001) [Psi(+)] prion generation in yeast: characterization of the 'strain' difference. *Yeast* **18**: 489-497.

Krishnan, R., and Lindquist, S.L. (2005) Structural insights into a yeast prion illuminate nucleation and strain diversity. *Nature* **435**: 765-772.

Kushnirov, V.V., Kryndushkin, D.S., Boguta, M., Smirnov, V.N., and Ter-Avanesyan, M.D. (2000) Chaperones that cure yeast artificial [PSI(+)] and their prion-specific effects. *Curr Biol* **10**: 1443-1446.

Tanaka, M., Chien, P., Naber, N., Cooke, R., and Weissman, J.S. (2004) Conformational variations in an infectious protein determine prion strain differences. *Nature* **428**: 323-328.

Tanaka, M., Chien, P., Yonekura, K., and Weissman, J.S. (2005) Mechanism of cross-species prion transmission: an infectious conformation compatible with two highly divergent yeast prion proteins. *Cell* **121**: 49-62.

Tanaka, M., Collins, S.R., Toyama, B.H., and Weissman, J.S. (2006a) The physical basis of how prion conformations determine strain phenotypes. *Nature* **442**: 585-589.

Tanaka, M., Collins, S.R., Toyama, B.H., and Weissman, J.S. (2006b) The physical basis of how prion conformations determine strain phenotypes. *Nature* **442/3**: 585-589.

Toyama, B.H., Kelly, M.J., Gross, J.D., and Weissman, J.S. (2007) The structural basis of yeast prion strain variants. *Nature* **449**: 233-237.

Toyama, B.H., and Weissman, J.S. (2011) Amyloid structure: conformational diversity and consequences. *Annu Rev Biochem* **80**: 557-585.

Uptain, S.M., Sawicki, G.J., Caughey, B., and Lindquist, S. (2001) Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion. *Embo J* **20**: 6236-6245.

Zhou, P., Derkatch, I.L., Uptain, S.M., Patino, M.M., Lindquist, S., and Liebman, S.W. (1999) The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. *EMBO J* **18**: 1182-1191.

Zhou, P., Derkatch, I.L., and Liebman, S.W. (2001) The relationship between visible intracellular aggregates that appear after overexpression of Sup35 and the yeast prion-like elements [PSI(+)] and [PIN(+)]. *Mol Microbiol* **39**: 37-46.

VITAE

NAME:	Jaya Sharma
EDUCATION:	B.S., Microbiology, National College, Tribhuvan University, Kathmandu, Nepal, 2001
	M.S., Clinical Microbiology, Tribhuvan University, Kathmandu, Nepal, 2004
	Ph.D., Biological Sciences, University of Illinois at Chicago, Illinois, 2013
TEACHING EXPERIENCE:	Teaching Assistant, Mendelian and Molecular Genetics, Department of Biological Sciences, University of Illinois at Chicago, IL: (Fall 2008, Spring 2009, Fall 2010, Spring 2012)
	Teaching Assistant, Microbiology Laboratory, Department fo Biological Sciences, University of Illinois at Chicago, IL: (Spring 2008, Summer 2008, Fall 2011)
	Teaching Assistant, Comparative Vertebrate Anatomy, Department of Biological Sciences, University of Illinois at Chicago, IL: (Fall 2007)
	National College, Tribhuvan University, Kathmandu, Nepal, 2004-2006
EXPERIENCE	Master's Thesis Research (Study of microbiology of upper respiratory tract infection with special interest in multi drug resistant and extended spectrum β-lactamase strains) Tribhuvan University, Kathmandu, Nepal 2003-2004
HONORS:	Academic Excellence Award (Mahendra Vidya Bhusan), M.S., Clinical Microbiology (University First rank), Tribhuvan University, Kathmandu, Nepal, 2004

ABSTRACTS:

Sharma J and Liebman S. W. (2009) When are the prion variants established? *3rd annual Midwest yeast meeting, Northwestern University, Evanston, IL*.

Sharma J and Liebman S. W. (2010). Investigation of [PSI+] Prion Variant Establishment in Yeast. *2010 FASEB Summer Research Conference, Protein Folding in the Cell. Saxtons River, VT.*

Sharma J and Liebman S. W. (2010). Investigation of [PSI+] Prion Variant Establishment in Yeast. *4th annual Midwest yeast meeting, Northwestern University, Evanston, IL.*

Sharma J and Liebman S. W. (2011). Investigation of [PSI+] Prion Variant Establishment in Yeast. *16 th Annual Midwest Stress Response and Molecular Chaperone Meeting, Northwestern University, Evanston, IL.*

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