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Diversity and Biogenesis of Yeast Prions

by

Lev Z. Osherovich

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

Lev Z. Osherovich

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Abstract

Prions are self-propagating, infectious protein aggregation states. Whereas in mammals, prions are associated with neurodegenerative illness, in fungi the prion mechanism has been co-opted to regulate intracellular protein activity. The yeast *Saccharomyces cerevisiae* harbors several proteins that can interconvert between soluble and aggregated forms; when aggregated, these prion states interfere with normal activity by other molecules of this type and are inherited in a non-Mendelian manner.

The prion-forming properties of the yeast protein Sup35p are conserved across millions of years of evolution, suggesting an adaptive function for the prion mechanism. Mutational analysis has revealed that aggregation (and prion formation) is mediated by a glutamine- and asparagine-rich tract at the amino terminus of the protein. Homology searches within the *S. cerevisiae* genome have lead to the discovery of an additional prion forming protein, New1p.

The amino-terminal region of New1p can also interconvert between soluble and aggregated forms, with distinct sequence determinants influencing its preferential specificity for aggregation with molecules of its own type as well as general determinants of prion heritability. Additionally, the presence of New1p aggregates renders cells susceptible to the aggregation of other glutamine- and asparagine-rich proteins, including Sup35p and glutamine-expanded human disease protein.

Thus, the prion behavior of New1p illustrates several principles of evolution and cell biology. First, the presence of multiple prion-forming proteins in the cell indicates adaptive and independent roles for various prions. Next, the differences and similarities of New1p and other prion-forming proteins shed light on the underlying mechanism of prion formation and propagation. Finally, the effect of New1p prions on the cellular physiology of protein aggregation points to broader mechanisms of intracellular homeostasis and quality control, bridging the fields of fungal genetics and human polyglutamine aggregation disease.

Jonathan Weissman

Jonathan S. Weissman, Ph.D.
Advisor

Acknowledgement of Contributions

Portions of the text in this dissertation are reproduced with permission from material published elsewhere. Chapter 1, "The Utility of Prions," was published as a review in *Developmental Cell*, February 2002, Vol. 2, pp. 143-151. Figure 1 was adapted from a drawing by Angela DePace. Figure 2 contains data adapted from Michelitsch and Weissman, *Proc. Natl. Acad. Sci.*, October 24, 2000, Vol. 97, pp. 7841-7846. Chapter 2 consists of "Molecular Basis of a Yeast Prion Species Barrier," published in *Cell*, January 21, 2000, Vol. 100, pp. 277-288, and is the result of a collaboration with Alex Santoso and Peter Chien. The work depicted in Figure 1 was performed by Mr. Chien, while the work in Figures 3, 4, 5 and 6 was performed by Dr. Santoso. Dr. Jonathan S. Weissman assisted in some of the strain construction described in Chapter 4, "Multiple Gln/Asn-Rich Prion Domains Confer Susceptibility to Induction of the Yeast [*PSI*$^+$] Prion," which was published in *Cell*, July 27, 2001, Vol. 106, pp. 183-194. With the above exceptions, the work presented in this dissertation was performed by its author, Lev Z. Osherovich, under the supervision of Dr. Jonathan S. Weissman.

Jonathan Weissman

Jonathan S. Weissman, Ph.D.

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Dear Dr Osherovich

CELL, Vol 100, 2000, pp 277-288, Santoso et al, "Molecular basis..."
Vol 27, 2001, pp 183-194, Osherovich et al, "Multiple Gln/Asn-Rich..."
DEVELOPMENTAL CELL, Vol 2, 2002, pp 143-151, Osherovich et al, "The utility of..."

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Chapter I: The Utility of Prions

Abstract

Infectious, self-propagating protein aggregates (prions), as well as structurally related amyloid fibrils, have traditionally been associated with neurodegenerative diseases in mammals. However, recent work in fungi indicates that prions are not simply aberrations of protein folding, but are in fact widespread, conserved and, in certain cases, apparently beneficial. Analysis of prion behavior in yeast has led to insights into the mechanisms of prion appearance and propagation, as well as the effect of prions on cellular physiology and perhaps evolution. The prion-forming proteins of *Saccharomyces cerevisiae* are members of a larger class of Gln/Asn-rich proteins that is abundantly represented in the genomes of higher eukaryotes, raising the prospect of genetically programmed prion-like behavior in other organisms.

Origins of the prion hypothesis: prions and self-replicating protein aggregates in human disease

The concept of an infectious protein or prion was first developed to explain the propagation of a set of related transmissible spongiform encephalopathies (TSEs) (for reviews, see (Prusiner, 1998; Weissmann, 1999). The infectious agent appeared to be composed largely (if not entirely) of a single endogenous protein called PrP. Remarkably, PrP can inter-convert between two states, the normal form (termed PrP^{c}) and its infectious variant (PrP^{Sc}). PrP^{c} has high α-helical content, whereas PrP^{Sc} adopts a β-sheet-rich conformation and in some cases aggregates to form long polymers termed amyloid fibers. These findings led to the "protein only" hypothesis in which replication of the infectious agent results from the ability of aggregated PrP^{Sc} to bind and catalyze conversion of PrP^{c} to PrP^{Sc}.

Despite concerns about the spread of Mad Cow disease (Collinge, 1999), TSEs remain extremely rare in humans. Prion-like amyloid protein aggregates are, however, intimately associated with a number of more common non-transmissible human diseases (Dobson, 1999). These include a variety of sporadic conditions such as the systemic amyloidoses as well as relatively common

neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Protein aggregation also underlies the dominantly inherited polyglutamine (polyGln) expansion disorders, which include Huntington's disease (HD) and Machado-Joseph disease (MJD) (Perutz, 1999; Zoghbi and Orr, 2000). Each of these syndromes involves the aggregation of a different protein but the aggregates themselves share a common β-sheet-rich amyloid structure. Moreover, these amyloids typically have a prion-like ability to recruit and catalyze the conversion of the normal cellular forms of the protein.

Prions as heritable regulators protein function: [*URE3*] and [*PSI*$^+$]

In 1994, Wickner (1994) extended the prion concept to explain the inheritance of two enigmatic non-Mendelian elements in the yeast *Saccharomyces cerevisiae*, called [*URE3*] and [*PSI*$^+$] (for reviews, see (Serio and Lindquist, 1999; Wickner et al., 2000). Unlike in mammals, yeast prions do not cause cell death, and can under certain circumstances even enhance survival. The phenotypes associated with [*URE3*] or [*PSI*$^+$] elements are not particularly exceptional: [*URE3*] alters nitrogen catabolite uptake

(Lacroute, 1971), while [*PSI*$^+$] increases the read-through of certain stop codons during translation (Cox, 1965). Indeed, traditional loss of function mutations in the chromosomally encoded nitrogen catabolism repressor Ure2p and the translation termination factor Sup35p mimic the [*URE3*] and [*PSI*$^+$] states, respectively. What make [*URE3*] and [*PSI*$^+$] so remarkable are their epigenetic properties; they are inherited by all of the meiotic progeny of diploid cells and can be transmitted by transfer of cytoplasm from one cell to another without the exchange of genetic material.

To explain the unusual inheritance of [*URE3*] and [*PSI*$^+$], Wickner proposed that these states result from the presence of self-propagating conformations or prions of the Ure2p and Sup35p proteins, respectively (see Figure 1). This model provides a mechanism for the non-nuclear inheritance and faithful propagation of the [*URE3*] and [*PSI*$^+$] states; the prion forms can convert free molecules of the affected protein and are themselves distributed along with the cytoplasm to all of the cell's progeny. The prion model also explains why the phenotypes of [*URE3*] and [*PSI*$^+$] mimic the loss of function of Ure2p and Sup35p, as conversion inactivates the affected protein.

A number of elegant experiments have established that [*URE3*] and [*PSI*$^+$] result from self-propagating aggregates of the Ure2p and

Sup35p proteins. Ure2p or Sup35p, which are ordinarily soluble, form protease-resistant aggregates in cells that are [*URE3*] or [*PSI*$^+$] (Masison and Wickner, 1995; Patino et al., 1996; Paushkin et al., 1996). The replication of Ure2p and Sup35p aggregates has been recapitulated in vitro both in cell extracts and with pure protein (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997; Taylor et al., 1999; Thual et al., 1999). For example, upon dilution from denaturant, Sup35p slowly forms amyloid fibers; this conversion can be greatly accelerated by the addition of a small amount of pre-formed Sup35p fibers. This "seeding" effect lies at the heart of [*PSI*$^+$] prion replication; mutations in Sup35p that either enhance or inhibit propagation of [*PSI*$^+$] in vivo cause a corresponding increase or decrease in the

rate of Sup35p polymerization in vitro (DePace et al., 1998; Glover et al., 1997; Kochneva-Pervukhova et al., 1998; Liu and Lindquist, 1999). Finally, mutant Sup35p that cannot seed the polymerization of wild type protein in vitro also fails to convert cells to [*PSI*$^+$] when over-expressed or introduced into yeast by liposome fusion (Sparrer et al., 2000).

[*URE3*] and [*PSI*$^+$] are examples of a broader phenomenon of glutamine- and asparagine-rich (Gln/Asn-rich) protein aggregation of

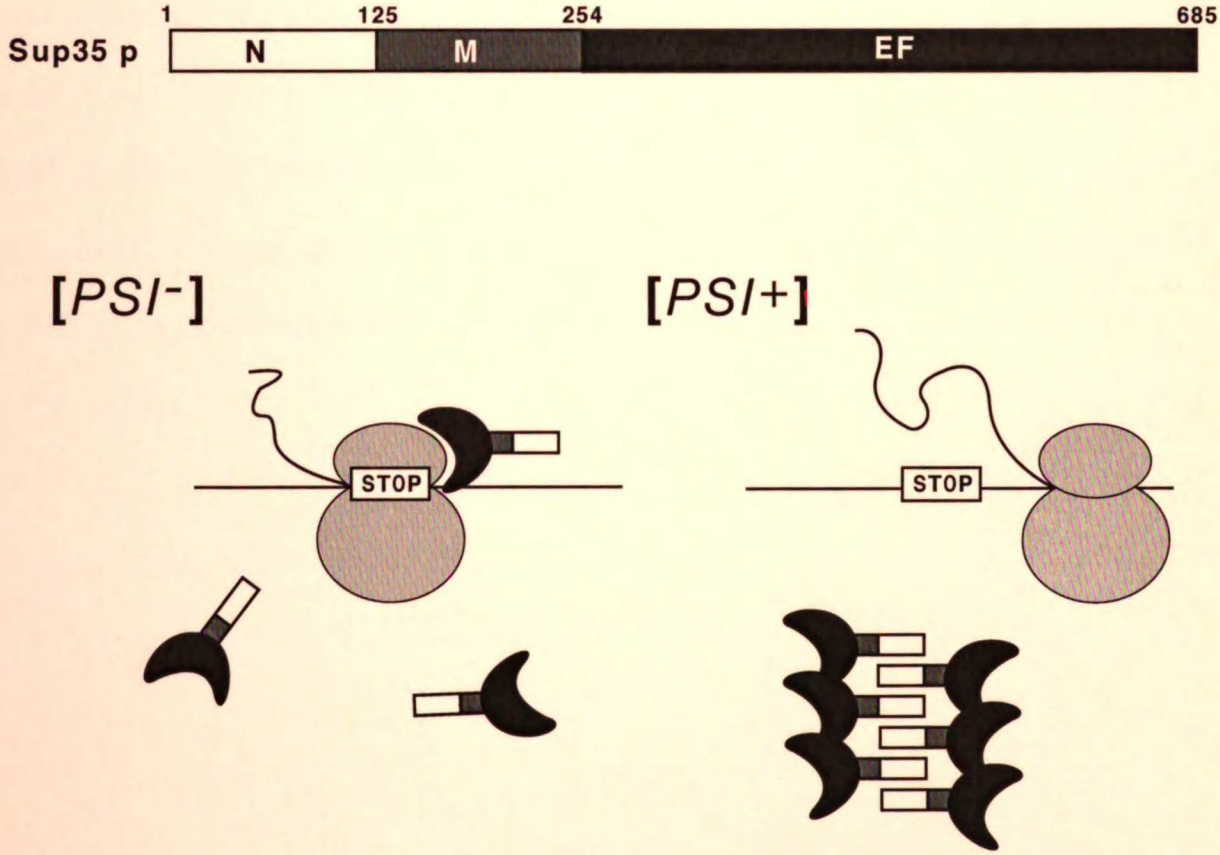
1
125
254
685
Sup35 p
N
M
EF
[PSI-]
[PSI+]
STOP
STOP

Figure 1. Prion model of [*PSI*$^+$]. Sup35p is a modular protein (top) consisting of an amino-terminal Gln/Asn-rich prion domain (N), a highly charged middle domain (M), and a conserved carboxy-terminal translation termination domain (EF). In [*psi*$^-$] yeast, Sup35p is soluble and functional, leading to robust translation termination at stop codons. In [*PSI*$^+$] yeast, Sup35p aggregates into a prion form via its N domain. Recruitment of newly made Sup35p allows propagation of the [*PSI*$^+$] prion while depletion of soluble Sup35p promotes the suppression of certain stop codons, leading to a selectable phenotype if an appropriate marker is used.

the kind implicated in human polyGln expansion disorders such as HD. The prion properties of Ure2p and Sup35p depend on Gln/Asn-rich domains at the amino termini of these proteins (DePace et al., 1998; Masison and Wickner, 1995; Parham et al., 2001; Paushkin et al., 1996; Ter-Avanesyan et al., 1993). These prion domains are otherwise dispensable for the normal functions of Ure2p and Sup35p and are modular, conferring the ability to aggregate when transferred to other proteins (Li and Lindquist, 2000; Patino et al., 1996). The structural basis of Gln/Asn-rich aggregation is thought to involve formation of "polar zippers" in which the β-sheets are stabilized by a network of hydrogen bonds involving the glutamine and asparagine side chains (Perutz et al., 1994). However, not every Gln/Asn-rich aggregate is a prion; in order to replicate and distribute itself among dividing cells, an aggregate may require additional structural features or interactions with other cellular factors.

Chaperones modulate prion inheritance in vivo

Although many Gln/Asn-rich proteins such as Sup35p and Ure2p have an intrinsic tendency to form self-replicating amyloid

aggregates in vitro, the propagation of yeast prions in vivo depends critically on a variety of chaperone proteins (Table 1). Certain chaperones that play a general role in ensuring proper protein folding, including members of the Hsp70 and Hsp40 families, inhibit prion formation and replication while other Hsp70 and Hsp40 chaperones actually promote prion propagation. Hsp104p, a chaperone that enhances thermotolerance by disaggregating heat-denatured proteins (Lindquist et al., 1995) does both; either an excess or a deficiency of Hsp104p results in the loss of [*PSI*$^+$] (Chernoff et al, 1995). The action of a particular chaperone typically varies from one prion to another; for example, while deletion of *HSP104* cures all known yeast prions, over-expression seems to cure only [*PSI*$^+$]. Likewise, over-expression of the Hsp40 protein Ydj1p efficiently cures [*URE3*] (Moriyama et al., 2000) but not [*PSI*$^+$]. The stable maintenance of prions may thus involve a balance between specific chaperone activities and the intrinsic tendency of prion aggregates to grow and divide.

Recent studies have begun to address how Hsp104p may function to enhance prion propagation. Hsp104p directly interacts with non-polymerized Sup35p in vitro (Schirmer and Lindquist, 1997), suggesting that excess Hsp104p could cure [*PSI*$^+$] either by

sequestering Sup35p or folding it into a state that is not susceptible to prion conversion. The requirement for endogenous levels of Hsp104p for the propagation of all yeast prions could result from the ability of this chaperone to break up large protein aggregates (Glover and Lindquist, 1998), thereby generating new prion seeds that are partitioned among dividing cells (Kushnirov and Ter-Avanesyan, 1998). Consistent with this view, Hsp104p is not required for the *de novo* Sup35p aggregation (Osherovich and Weissman, 2001; M. Tuite, personal communication), while depletion of Hsp104p activity in [*PSI*$^+$] cells decreases the number of Sup35p aggregates but increases their size (Wegrzyn et al., 2001). Additionally, the curing of prions by growth in the presence of guanidine hydrochloride, a small molecule that was recently shown to inhibit Hsp104p in vivo (Ferriera, et al., 2001; Jung and Masison, 2001), results from the inability of the prion seed to replicate. Efficient guanidine-mediated curing of [*PSI*$^+$] requires roughly seven cell divisions, regardless of how quickly these divisions occur; this finding has allowed Eaglestone et al. (2000) to estimate that a typical [*PSI*$^+$] cell has approximately 60 independent prion seeds.

Another example of the interplay of chaperone activities on prions comes from the opposing effects of different Hsp70 proteins on [*PSI*$^+$] (see Table 1). One family of these ubiquitous cytosolic

Family	Yeast Protein	Prion	Effect	Citation
HSP100	Hsp104p	[*PSI*$^+$]/[*ETA*$^+$]	Δ: cures	Chernoff et al. 1995 Zhou et al. 1999
	" "	" "	↑: cures	" "
	" "	[*PSI*$^+_{ps}$]	Δ: cures	Kushnirov et al. 2000b
	" "	" "	↑: no effect	" "
	" "	[*PSI*$^+_{CA}$]/[*CHI*$^+_{CA}$]	Δ: cures	Santoso et al. 2000
	" "	" "	↑: cures	" "
	" "	[*PIN*$^+$]/[*RNQ*$^+$]	Δ: cures	Derkatch et al. 1997
	" "	" "	↑: no effect	" "
	" "	[*URE3*]	Δ: cures	Moriyama et al. 2000
	" "	" "	↑: no effect	" "
	" "	[*NU*$^+$]	Δ: cures	Osherovich and Weissman 2001
	" "	" "	↑: no effect	Osherovich and Weissman (unpublished data)
HSP70	Ssa1p/Ssa2p	[*PSI*$^+$]	mutant alleles cure*	Jung et al. 2000
	" "	" "	↑: antagonizes curing by Hsp104p over-expression; enhances prion phenotype	Newnam et al. 1999
	" "	[*PSI*$^+_{ps}$]	↑: cures (enhanced by Ydj1p co-over-expression)	Kushnirov et al. 2000b
	" "	[*PIN*$^+$]/[*RNQ*$^+$]	Interacts with the prion form of Rnq1p	Sondheimer et al. 2001
	Ssb1p/Ssb2p	[*PSI*$^+$]	Δ: antagonizes curing by Hps104p over-expression; increases prion appearance	Chernoff et al. 1999
	" "	" "	↑: cures or enhances curing by HSP104 over-expression	Chernoff et al. 1999 Chacinska et al. 2001
	" "	[*PSI*$^+_{ps}$]	↑: cures	Kushnirov et al. 2000b
HSP40	Ydj1p	[*PSI*$^+_{ps}$]	↑: cures (enhanced by SSA co-over-expression)	" "
	" "	[*URE3*]	↑: cures	Moriyama et al. 2000
	Sis1p	[*PIN*$^+$]/[*RNQ*$^+$]	Deletion of the C-terminal domain cures; other mutations affect prion aggregate morphology; interacts with prion form of protein	Sondheimer et al. 2001
Small HSPs	Hsp26	[*PSI*$^+$]	↑: no effect	M. Tuite (pers. comm.)
	" "	" "	Δ: no effect	" "

Table 1. Summary of the relationships between chaperones and yeast prions. Δ indicates deletion, ↑ indicates over-expression. Prions are listed with their closely related variants or alternative names. [PSI^{+}_{ps}] is a prion form of the *Pichia methanolica* Sup35p, while [PSI^{+}_{CA}]/[CHI^{+}_{CA}] is that of *Candida albicans*. *: When both SSA1 and SSA2 are deleted, the homologous *SSA3* and *SSA4* genes are induced, thus allowing [*PSI*$^{+}$] propagation. Jung et al.(2000) obtained an allele of *SSA1* that, in combination with SSA2 deletion, does not induce *SSA3*/*SSA4* and results in the loss of [*PSI*$^{+}$].

chaperones, the SSA proteins, appears to promote the aggregation of Sup35p; over-expression of SSA proteins increases the extent of Sup35p aggregation and antagonizes the curing of [*PSI*$^+$] by *HSP104* over-expression (Newnam et al., 1999), whereas depletion of SSA activity promotes the loss of [*PSI*$^+$] (Jung et al., 2000). Conversely, another family of Hsp70 proteins, encoded by *SSB1* and *SSB2*, antagonizes Sup35p aggregation; over-expression of SSB proteins enhances curing of [*PSI*$^+$] while deletion has the opposite effect (Chernoff et al., 1999; Chacinska et al., 2001). As SSB chaperones are thought to act upon nascent proteins during translation, it is possible that the conversion of Sup35p to a prion state may occur during the protein's synthesis, before it has adopted a final stable conformation.

Conservation and utility of [*PSI*$^+$]

Several observations suggest that [*PSI*$^+$] prions are conserved and may even serve important biological functions. [*PSI*$^+$] is found in many laboratory strains, even in the absence of specific selection for stop codon read-through. If the [*PSI*$^+$] state provided no benefit, one might expect that the ability to form it would be rapidly lost, as mutational studies have found that even single point mutations

within the Sup35p prion domain are sufficient to dramatically inhibit [*PSI*$^+$] propagation in vivo (DePace et al., 1998; Doel et al., 1994). However, analysis of the amino-terminal domains of Sup35p from a variety of budding yeast species separated by many millions of years of evolution revealed the conservation of the fundamental properties of these proteins (Chernoff et al., 2000; Kushnirov et al., 2000; Kushnirov et al., 1990; Santoso et al., 2000). While the specific sequences of these prion domains are rather divergent, the amino-terminal portions of Sup35p from all of the examined species share a high Gln/Asn content as well as a set of imperfect oligopeptide repeats, features shown by mutational analysis to be critical for prion propagation.

When transplanted into *S. cerevisiae*, the Gln/Asn-rich domains of Sup35p from other yeasts could reversibly aggregate into [*PSI*$^+$]-like states, the formation and propagation of which typically displayed a high degree of specificity for proteins of their own kind. For example, in a strain expressing the amino-terminal domains of Sup35p from both *S. cerevisiae* and *Candida albicans*, each protein formed a prion that propagated independently of the state of the other protein. This specificity resembles the "species barrier" that ordinarily prevents interaction between PrP prions from different animals. The Sup35p prion domains from different yeasts have

allowed several groups to study the basis and limitations of prion specificity in vivo and in vitro (Chien and Weissman, 2001; King, 2001; Nakayashiki et al., 2001). Recently, Nakayashiki et al. (2001) have reported a [*PSI*$^+$]-like phenomenon with an associated translation termination defect in *Kluyveromyces lactis* that results from prion formation by the homologue of Sup35p. Thus, it seems that in addition to the prion-forming Gln/Asn-rich domain, the cellular machinery needed for [*PSI*$^+$] propagation is conserved in other yeasts.

Why are the prion properties of Sup35p conserved? Perhaps natural selection favors yeasts that can switch the activity of this protein on and off in an epigenetically heritable manner. Two groups have examined whether [*PSI*$^+$] can confer a selective advantage under conditions that resemble those encountered by yeast in the wild. Eaglestone et al. (1999) found that [*PSI*$^+$] strains exhibited an elevated tolerance to heat stress and ethanol as a result of their defective translation termination. True and Lindquist (2000) compared [*PSI*$^+$] isolates of a number of different genetic backgrounds with their prion-free (or [*psi*$^-$]) derivatives, and found that under a number of growth conditions certain yeast strains grew better if they were [*PSI*$^+$], while many other conditions favor [*psi*$^-$] cells. These observations led to the interesting proposal that [*PSI*$^+$]

may act as an "evolutionary capacitor" (Rutherford and Lindquist, 1998) which allows previously neutral genetic variation to convert to a non-neutral state thereby facilitating the evolution of new traits (True and Lindquist, 2000). De novo appearance of yeast prions can be stimulated by prolonged incubation at low temperature (Chernoff et al. 1995, Derkatch et al 2000, Y.O. Chernoff, personal communication), while the reversion of [*PSI*$^+$] to [*psi*$^-$] can be triggered by chemical agents such as guanidine (Tuite et al., 1981). Thus, although the ability to form and propagate the [*PSI*$^+$] prion must have resulted from natural selection, the environmental responsiveness and epigenetic properties of the prion itself are reminiscent of Lamarckian "inheritance of acquired characteristics" (Chernoff, 2001).

Prediction and identification of novel prions

The discovery of conserved and perhaps adaptive properties of [*PSI*$^+$] raises the question of whether prion forms of proteins other than Sup35p and Ure2p exist. A surprisingly large number of Gln/Asn-rich sequences reminiscent of the prion domains of Ure2p and Sup35p can be predicted from eukaryotic genomes including those of *S. cerevisiae*, *C. elegans* and *D. melanogaster* (Figure 2)

(Michelitsch and Weissman, 2000). Initial efforts to test prion formation by these proteins concentrated on yeast because of the ease of genetic analysis (Santoso et al., 2000; Sondheimer and Lindquist, 2000). As it was difficult to anticipate the prion-associated phenotypes for these proteins, many of which were non-essential or poorly characterized, both we and Sondheimer and Lindquist made use of the easily assayed translation termination phenotype of [*PSI*$^+$]. To test for prion formation by candidate sequences, these were swapped into the *SUP35* gene to replace the endogenous prion domain; the resulting chimeric proteins were then tested for the ability to form a [*PSI*$^+$]-like state after transient over-expression of the putative prion protein in question.

Using this "artificial prion" method, two previously uncharacterized proteins (New1p and Rnq1p) were found to contain prion domains. A fusion of the first 153 residues of New1p and the translation domain of Sup35p could inter-convert between two states, termed [*nu*$^-$] and [*NU*$^+$], which respectively resulted from soluble and aggregated forms of the chimeric protein. Similarly, a Sup35p fusion with the carboxy-terminal portion of Rnq1p could reversibly form an aggregated state termed [*RPS*$^+$]. The full length Rnq1p also appeared to form a genuine endogenous prion in a number of "wild type" yeast strains, as it was found in a high

molecular weight complex that could be permanently disrupted by treatment of the cells with guanidine. However, no obvious phenotype associated with the Rnq1p prion was initially evident.

A different line of experimentation had earlier uncovered a prion-like epigenetic element with surprising properties. Derkatch et al. (1997) described a cryptic epigenetic state called [*PIN*$^+$] (standing for PSI INducibility) that influenced the cell's susceptibility to the *de novo* induction of [*PSI*$^+$]; only strains that possessed the [*PIN*$^+$] trait could readily convert to [*PSI*$^+$] upon over-expression of Sup35p. [*PIN*$^+$] had all the hallmarks of a prion, including cytoplasmic inheritance, reversible curing and dependence on cellular chaperones. However, propagation of [*PIN*$^+$] did not require Sup35p and was independent of [*PSI*$^+$], suggesting that [*PIN*$^+$] was a naturally occurring prion form of some other protein. This conclusion was confirmed by the subsequent identification of Rnq1p as the protein behind [*PIN*$^+$] (Derkatch et al., 2001).

Prions affect the aggregation of other proteins

Surprisingly, Rnq1p is only one of a large number of proteins that, when aggregated, can render a cell susceptible to [*PSI*$^+$] induction (Derkatch et al., 2001; Osherovich and Weissman, 2001). To identify the protein(s) responsible for the [*PIN*$^+$] phenomenon,

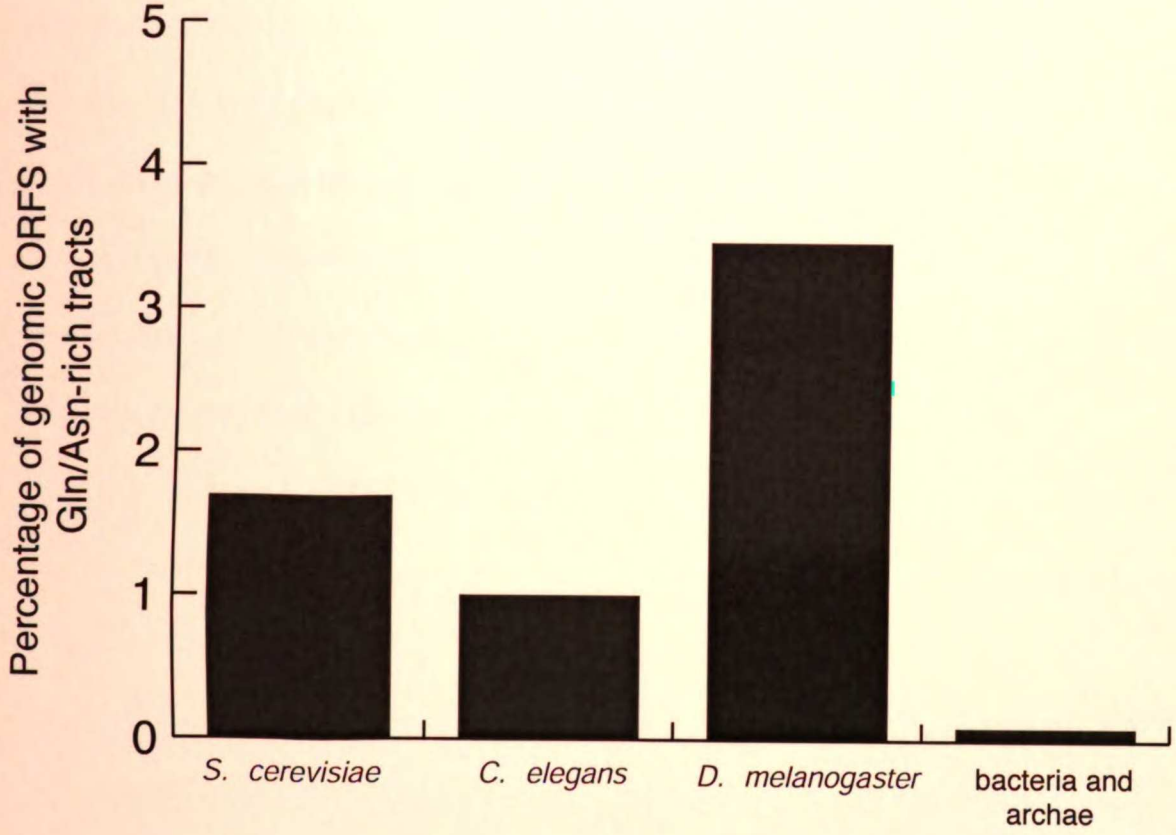
Percentage of genomic ORFS with Gln/Asn-rich tracts
5
4
3
2
1
0
S. cerevisiae
C. elegans
D. melanogaster
bacteria and archae

Figure 2. Gln/Asn-rich tracts are abundant in certain eukaryotic proteomes. (Michelitsch and Weissman, 2000). The complete genomic sequences of three eukaryotes as well as of twenty bacteria and archae were searched for Gln/Asn-rich tracts, defined as regions of open reading frames (ORFs) with 30 or more Gln and Asn residues per 80-residue window. The frequency of such sequences in the eukaryotes is much greater than expected if amino acid composition were due to chance. Interestingly, the recently sequenced human genome has a lower abundance (~0.3%) of predicted Gln/Asn-rich tracts than other metazoans (M. Michelitsch, personal communication).

Derkatch et al. (2001) reasoned that over-expression of the appropriate prion-forming protein might spontaneously induce a [*PIN*$^+$]-like state. A screen for [*PSI*$^+$]-promoting high-copy plasmids yielded *NEW1*, *URE2*, and nine other genes, all of which encoded proteins with Gln/Asn-rich domains. Concurrently, a candidate gene approach (Osherovich and Weissman, 2001) demonstrated that over-expression of the Gln/Asn-rich domains of New1p and Rnq1p obviated the requirement for [*PIN*$^+$] in the process of [*PSI*$^+$] induction. In each case, it was specifically the aggregated form of the protein in question that made cells susceptible to the induction of [*PSI*$^+$]. For example, the prion form of New1p, [*NU*$^+$], promoted [*PSI*$^+$] induction at an efficiency comparable to that of the endogenous [*PIN*$^+$] prion.

This epigenetic regulation of protein aggregation extends beyond [*PSI*$^+$] induction and even beyond prions. The prion-promoting relationship between aggregation-prone proteins is reciprocal; the presence of Sup35p aggregates (i.e. [*PSI*$^+$]) allows other prion-forming proteins such as Ure2p and Rnq1p to themselves form aggregates (Derkatch et al., 2001). Furthermore, the aggregation of polyGln-expanded MJD protein in yeast was greatly enhanced by the presence of aggregation-promoting prions such as [*PIN*$^+$] and [*NU*$^+$] (Osherovich and Weissman, 2001),

suggesting the possible relevance of prion-facilitated aggregation to human disease.

The screen for prion-promoting proteins developed by Derkatch et al. (2001) also provides a potential method for identifying novel prion-like elements. The genes identified in this screen include a number of interesting candidates such as a casein kinase (Yck1p), an Sm-like protein (Lsm4p) and a well-characterized chromatin remodeling factor (Swi1p). The latter is part of a larger protein complex involved in global transcriptional activation (reviewed by Sudarsanam and Winston, 2000) that includes Swi3p, Snf2p, Snf5p, Snf6p, and Snf11p, all which contain Gln/Asn-rich tracts. While there is no evidence that components of the Swi/Snf complex form prions, it is possible that the Gln/Asn-rich sequences in these proteins stabilize interactions between them in a manner that resembles the β-sheet structure of amyloids.

How could prions promote the appearance of other protein aggregates? Any model must account for the fact that these prions act through a dominant, gain-of-function mechanism, rather than because of depletion of their normal protein activity. One possibility is that prions can directly seed the growth of other prions or aggregates (Figure 3A). For example, there is evidence for a low level of "cross-seeding" between certain Sup35p prions (Nakayashiki

et al., 2001). A related model (Figure 3B) postulates that the presence of prions induces or activates an enzymatic activity that itself promotes the aggregation of other proteins; for example, one type of prion could recruit and concentrate chaperones, thereby providing a surface for the assembly of other prions. In both of these models, the aggregate-promoting prions have a stimulatory effect on a process that ordinarily is unfavorable.

An alternative model proposes that aggregation-prone proteins are prevented from establishing stable aggregates by the action of an inhibitory factor that can be inactivated by the presence of prions (Figure 3C). Consistent with this model, de novo aggregation appears to be inhibited in prion-free cells; for example, Sup35p spontaneously forms aggregates in vitro at very low concentrations, but even strong over-expression of Sup35p in [*pin*$^-$] cells causes neither [*PSI*$^+$] induction nor the appearance of detectable Sup35p aggregates. Moreover, extracts prepared from yeast strains lacking Hsp104p, and thus any endogenous prions such as [*PIN*$^+$], inhibit the spontaneous aggregation of polyGln-expanded huntingtin protein to a greater degree than do wild type extracts (Cao et al., 2001). A similar inhibitory effect of extracts on Sup35p polymerization in vitro has recently been observed by Uptain et al. (2001). However, genetic screens for mutations that lead to phenotypic emulation of

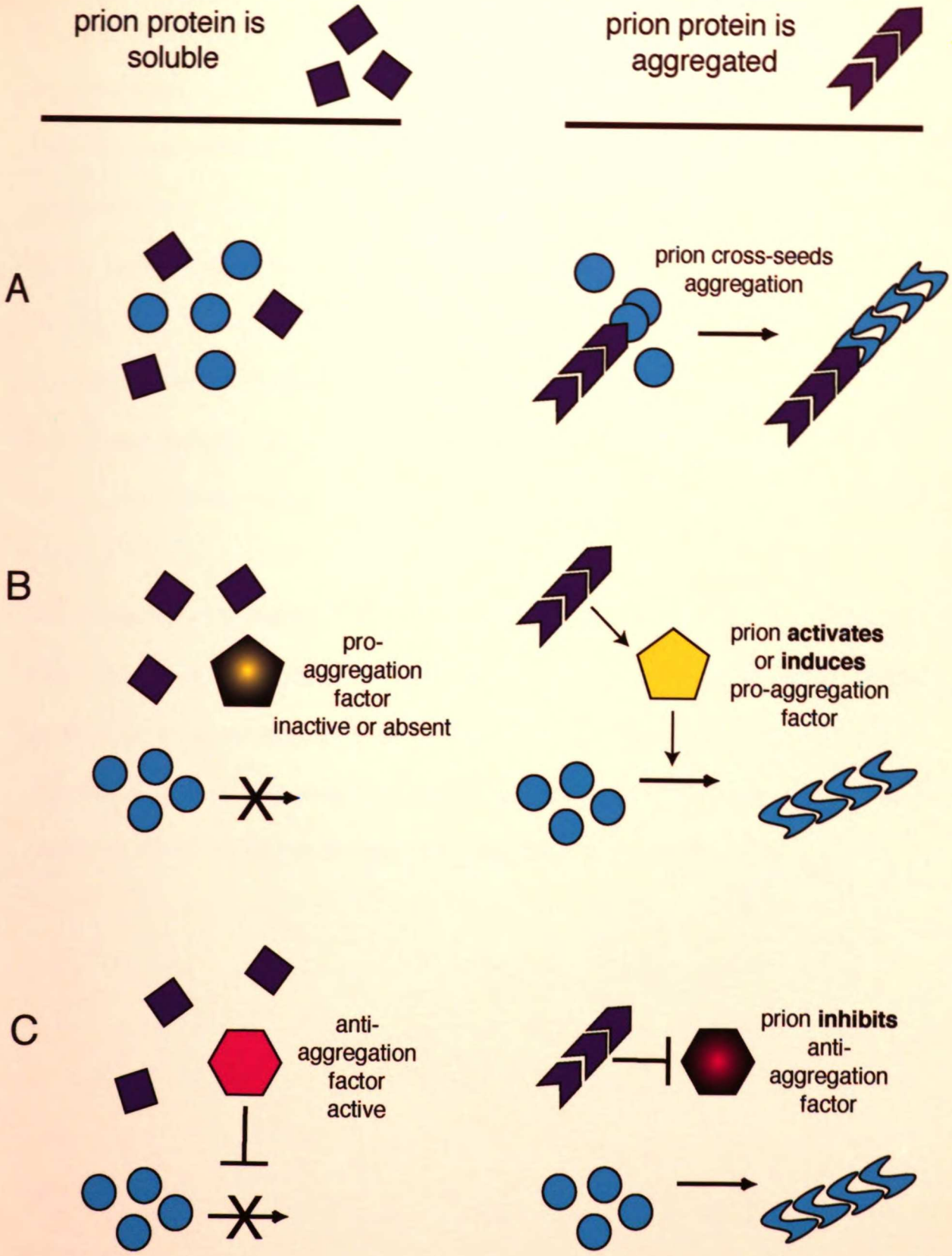
prion protein is soluble
prion protein is aggregated
A
prion cross-seeds aggregation
B
pro-aggregation factor inactive or absent
prion **activates** or **induces** pro-aggregation factor
C
anti-aggregation factor active
prion **inhibits** anti-aggregation factor

Figure 3. Three hypothetical models for prion-facilitated initiation of aggregation.

(A) **Cross-seeding**. In this model, multiple aggregation-prone proteins can directly interact with one another. For example, the prion form of one protein (purple) seeds the aggregation of another (blue).

(B) **Stimulation of aggregation.** Prions (purple) activate or induce the expression of a protein or complex (yellow), which then promotes the conversion of another protein (blue) to an aggregated form.

(C) **Inhibition of anti-aggregation**. An aggregation-prone protein (blue) is prevented from aggregating or is disaggregated by the action of an inhibitory protein or complex (pink). However, prions (purple) can interact with and inactivate this anti-aggregation factor, perhaps by acting as competitive inhibitors. The blue protein is then free to aggregate.

[*PIN*$^+$] by inactivating inhibitors of prion formation have thus far been unsuccessful (Derkatch et al., 2001). One potential target of [*PIN*$^+$] elements, which might be difficult to identify genetically due to its essential role in protein metabolism, is the ubiquitin-proteasome machinery. In mammalian cells, the proteasome can be saturated by Gln/Asn-rich aggregates (Bence et al., 2001); this could prevent the degradation of aggregation-prone proteins, leading to their accumulation and eventual conversion to prions or other aggregates.

Normal protein activity by prions

Prions of mammals and yeast have traditionally been thought of as aberrant protein states that interfere with protein function or cause toxicity. However, recent work on the filamentous fungus *Podospora anserina* has unexpectedly uncovered a role for prions in the "normal" biology of this organsim.

Many filamentous fungi, including *P. anserina,* grow as haploid syncitial networks termed mycelia. When two mycelia encounter each other, they can fuse and grow as a single network, called a heterokaryon, with nuclei and cytoplasm from both parent colonies. However, stable heterokaryon propagation occurs only if both partners have identical alleles at a number of genetic "heterokaryon

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incompatibility" loci (Saupe et al., 2000). There are nine such *het* loci in *P. anserina*. One of these, the S locus, has two principle allelic variants that are denoted in upper and lower case and encode proteins that differ from one another by thirteen residues. When a *het-S* strain fuses with an incompatible *het-s* strain, the resulting heterokaryon rapidly dies and becomes cut off from the rest of the mycelium.

The prion connection emerged from the discovery that genetically identical *het-s* strains can exist in two states, a functional form called [Het-s] and an inactive variant called [Het-s*] (Coustou et al., 1997). The *het-s* gene is the same in both [Het-s] and [Het-s*] strains, but when the two strains form a heterokaryon, the [Het-s] state is dominant; furthermore, the [Het-s] form spreads rapidly through the recipient [Het-s*] mycelium and can in certain cases be passed down to all of the haploid progeny of a sexual cross. Propagation of the [Het-s] state requires a 25-residue N-terminal sequence of the HET-s protein (Coustou et al., 1999). Finally, the HET-s protein is aggregated and resistant to proteolysis in cellular extracts from [Het-s] strains, and forms amyloid fibrils in vitro (Dos Reis et al., 2001). These data argue that the [Het-s] state results from prion formation by the HET-s protein. Rather than

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diminishing the activity of HET-s protein, the prion form here appears to be required for its normal physiological function.

Are there prions in other organisms?

A reasonable place to begin the search for prions in higher organisms is among the numerous Gln/Asn-rich proteins predicted from genomic analysis (Figure 2). As proteins with large numbers of Gln and Asn residues have a tendency to aggregate in vitro, it seems likely that at least some of them might support prion propagation when expressed in yeast. However, Gln/Asn content should not be the sole criterion used to search for novel prions, as neither PrP nor Het-s are particularly Gln/Asn-rich.

Even if prion-forming proteins from other organisms are identified using the yeast system, the real challenge would be to observe prions within their natural hosts. The best candidate genes could be transiently over-expressed in worms or flies and assayed for subsequent self-propagating aggregation. However, it is difficult to predict what effect any such prion states would have on the cell or the organism, especially if the function of the protein in question is unknown. Prions could inactivate the affected protein, as in [*PSI*$^+$], or may have dominant effects akin to the aggregation-

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promoting effects of [*PIN*$^+$] and [*NU*$^+$]. For example, prions might enhance the aggregation and deleterious effects of polyGln-expanded proteins in worms and flies, as they do in yeast.

Alternatively, one could look for prion phenomena in natural populations. One obstacle to this approach is the uncertainty of how prions would be transmitted in higher eukaryotes. Mammalian prions spread horizontally from one animal to another, while intracellular fungal prions are transmitted both vertically and horizontally through cell division and mating, respectively. Ostensibly, neither mechanism of infectivity is likely in most experimentally tractable organisms. Worms and flies do not generally eat each other and, because somatic cells undergo only a limited number of cell divisions, vertical transmission of intracellular prions would be limited. Since the maintenance of prions requires the continuous expression of the host protein, prion states might not be transmitted through the germ line and would thus not be detectable, as in fungi, through non-Mendelian inheritance.

Given the complexity of identifying prion phenotypes and subtleties of detecting prion inheritance, how could one spot conformational inheritance in multicellular organisms? The spontaneous aggregation of prion-forming proteins might poison the affected cells, either through loss of the protein's normal activity or

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as a result of toxicity of the aggregate itself; if this happens early enough in development, this might lead to the stochastic loss of certain cell lineages. Alternatively, if the affected cells do not die, the resulting animal would be a mosaic of normal and prion-containing cells. Such variegation could, of course, be detected only if there is a convenient phenotype to distinguish between the two cell types.

Finally, one could look for structural similarities between prions and complexes that are associated with epigenetically inherited phenomena such as centrosome replication, gene silencing and chromatin structure. In this light, the identification of the chromatin remodeling factor Swi1p as a prion-promoting protein (Derkatch et al. 2001) as well as the Gln-Asn-rich content of the Swi/Snf complex is particularly interesting.

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Chapter II. Identification of the Novel Prion-Forming Protein, New1p

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Molecular Basis of a Yeast Prion Species Barrier

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Summary

The yeast [PSI$^+$] factor is inherited by a prion mechanism involving self-propagating Sup35p aggregates. We find that Sup35p prion function is conserved among distantly related yeasts. As with mammalian prions, a species barrier inhibits prion induction between Sup35p from different yeast species. This barrier is faithfully reproduced in vitro where, remarkably, ongoing polymerization of one Sup35p species does not affect conversion of another. Chimeric analysis identifies a short domain sufficient to allow foreign Sup35p to cross this barrier. These observations argue that the species barrier results from specificity in the growing aggregate, mediated by a well-defined epitope on the amyloid surface and, together with our identification of a new yeast prion domain, show that multiple prion-based heritable states can propagate independently within one cell.

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Introduction

Amyloid protein aggregates have been increasingly implicated in human diseases, including prion-based encephalopathies, non-infectious neurodegenerative diseases, and systemic amyloidoses (Collinge, 1997; Koo et al., 1999). Amyloids are β-sheet rich, ordered structures consisting of protofibrils (Jiménez et al., 1999; Sunde and Blake, 1997) that coalesce in vitro to form extended fibrils which bind the dye Congo red. Fibrils are also found under some conditions in vivo, although their role in pathogenesis remains unresolved (Lansbury, 1999). Despite having similar aggregated structures, sequence comparison of amyloidogenic proteins fails to reveal any obvious similarities.

A striking property of most amyloids is the ability to catalyze their own propagation. In prion diseases, this self-propagation is thought to be the basis of protein-mediated infectivity. Here, the abnormal β-sheet rich prion form (PrP^{sc}) can convert the normal cellular α-helical protein (PrP^{c}) into the prion isoform (Prusiner, 1998; Weissmann, 1999). Even in the non-infectious amyloid diseases, such as Alzheimer's disease, amyloid self-propagation may be critical to disease progression (Lansbury, 1999).

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Studies of the mammalian prion have highlighted the importance of specificity in amyloid propagation. Here, a species barrier limits PrP^{SC} derived from one species from infecting another preventing, for example, the transmission of scrapie from sheep to man (Prusiner, 1998). The recent description of variant Creutzfeld-Jacob disease, however, suggests that in rare instances bovine prions can cross the species barrier to infect humans. Extensive transgenic mouse and chimera analyses indicate that the species barrier is largely due to differences in sequence of the prion proteins. However, the extent to which the species barrier is mediated by direct interaction between prion particles as opposed to species-specific interactions with cellular factors is unresolved (Kocisko et al., 1995; Telling et al., 1995). Even in non-prion amyloid diseases, the ability of amyloids to incorporate other types of protein has been implicated in the disease process (Han et al., 1995), although other studies have failed to observe cross-seeding between different amyloid forming peptides (Come et al., 1993).

The prion-like phenomenon [PSI^+] of *Saccharomyces cerevisiae* offers a powerful system to study the molecular basis of amyloid propagation and specificity. Identified as a non-Mendelian trait that confers suppression of nonsense mutations, [PSI^+] arises from conversion of the translational termination factor Sup35p from

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a soluble and active state into an insoluble and inactive amyloid (Liebman and Derkatch, 1999; Tuite and Lindquist, 1996; Wickner et al., 1995). The ability of Sup35p amyloids to incorporate newly made soluble proteins is thought to be the basis of [PSI$^+$] propagation. As Sup35p aggregation increases translational readthrough, the presence of the [PSI$^+$] prion can be readily monitored.

To investigate the requirements for prion formation and amyloid specificity and to examine if there is evolutionary pressure to retain prion function, we cloned and characterized Sup35p from a spectrum of Saccharomycetales (budding yeasts). Interestingly, we find that the ability to support a prion mechanism of inheritance is broadly conserved. Moreover, as with mammalian prions, a species barrier prevents cross-species prion induction. We have taken advantage of these phenomena to elucidate the requirements for prion formation and the molecular basis of this species barrier.

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Results

The Prion Domain is Conserved in Yeast Evolution

The N-terminus of Sup35p is necessary and sufficient for prion formation and propagation. This prion domain (PrD) is connected to the C-terminal translation termination domain (EF) by a highly charged middle domain (M) of unknown function (Stansfield et al., 1995; Ter-Avanesyan et al., 1993). Deletion of the PrD allows Sup35p to remain soluble and functional even in [PSI$^+$] yeast, whereas transient overexpression of this domain induces conversion of [psi$^-$] yeast to [PSI$^+$] (Patino et al., 1996; Paushkin et al., 1996). Mutational analyses have begun to define the sequence requirements for prion formation and propagation (DePace et al., 1998; Liu and Lindquist, 1999). Although the PrD is generally tolerant to amino acid changes, several unusual features were found to be important. In particular it has a high glutamine (Gln) and asparagine (Asn) and low charge content. In addition, the PrD has a set of imperfect oligopeptide repeats, deletion and expansion of which modulate its ability to induce the conversion to [PSI$^+$].

To determine whether these features are conserved, we cloned and characterized Sup35p PrDs from a variety of yeasts. Taking advantage of the conservation of the EF domain, we used

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one-sided PCR (Frohman, 1993) as well as available *SUP35* sequences ((Kushnirov et al., 1990) to clone sequences upstream of this region. In total, we examined *SUP35* genes from seven non-*S. cerevisiae* budding yeast species (*Candida albicans*, *Kluyveromyces lactis* and *marxianus*, *Pichia methanolica* and *pastoris*, *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxi*) (Figure 1A).

Significantly, all of the Sup35 proteins examined have N-terminal regions similar in composition to the PrD and M domains of the *S. cerevisiae* protein (Figures 1B and C). Although there is little exact sequence homology, all of the PrDs have a high Gln/Asn (36 to 43 percent) and a low charge (2 to 10 percent) content. This composition resembles that of a modular prion domain from another *S. cerevisiae* prion protein, Ure2p (Edskes et al., 1999), but is very different from that of full length proteins from the *S. cerevisiae* genome which have on average 9 percent Gln/Asn and 23 percent charged residues (Figure 1B). Finally, the imperfect oligopeptide repeats of QGGYQQYN, although highly divergent, are clearly detectable (Figure 1C).

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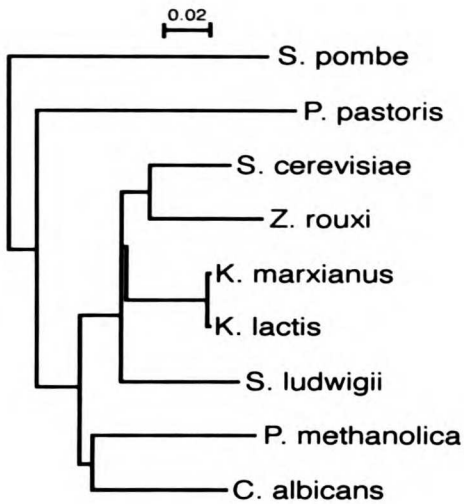
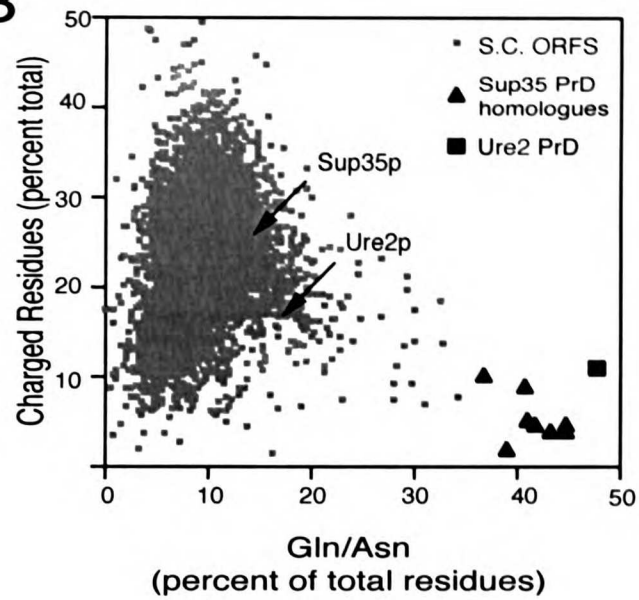
Foreign PrDs Aggregate, but do not Interact with *S. cerevisiae* Sup35p

The unusual sequence composition common to all Sup35p N-terminal domains prompted us to examine if the ability to support prion-based inheritance is also conserved. Focusing on *C. albicans*, *K. lactis* and *P. methanolica*, we asked whether the foreign Sup35p N-terminal domains could be recruited efficiently into the *S. cerevisiae* [PSI$^+$] aggregate using both functional and visual assays. In the functional assay, foreign PrDM fused to *S. cerevisiae* EF domain, termed PrDM-EF, is ectopically expressed in the [PSI$^+$] yeast. The PrDM-EF gene is under control of the *S. cerevisiae* *SUP35* promoter, resulting in a moderate, constitutive level of expression. If the foreign PrDs were not incorporated in the endogenous [PSI$^+$] aggregate, soluble fusion protein would provide functional translation termination activity, thereby leading to an antisuppressed phenotype. This can be phenotypically monitored by use of yeast harboring an *ade-1* marker with a suppressible nonsense mutation (Chernoff et al., 1995). In suppressed yeast, functional Ade1p is produced, resulting in white colonies on low adenine medium and growth on adenine-free medium. By contrast, the lack of functional Ade1p in either [psi$^-$] or antisuppressed [PSI$^+$] yeast results in red colonies on low adenine and lack of growth on

A


0.02
S. pombe
P. pastoris
S. cerevisiae
Z. rouxi
K. marxianus
K. lactis
S. ludwigii
P. methanolica
C. albicans

B


S.C. ORFS
Sup35 PrD homologues
Ure2 PrD
Sup35p
Ure2p
Charged Residues (percent total)
Gln/Asn
(percent of total residues)
0
10
20
30
40
50

C

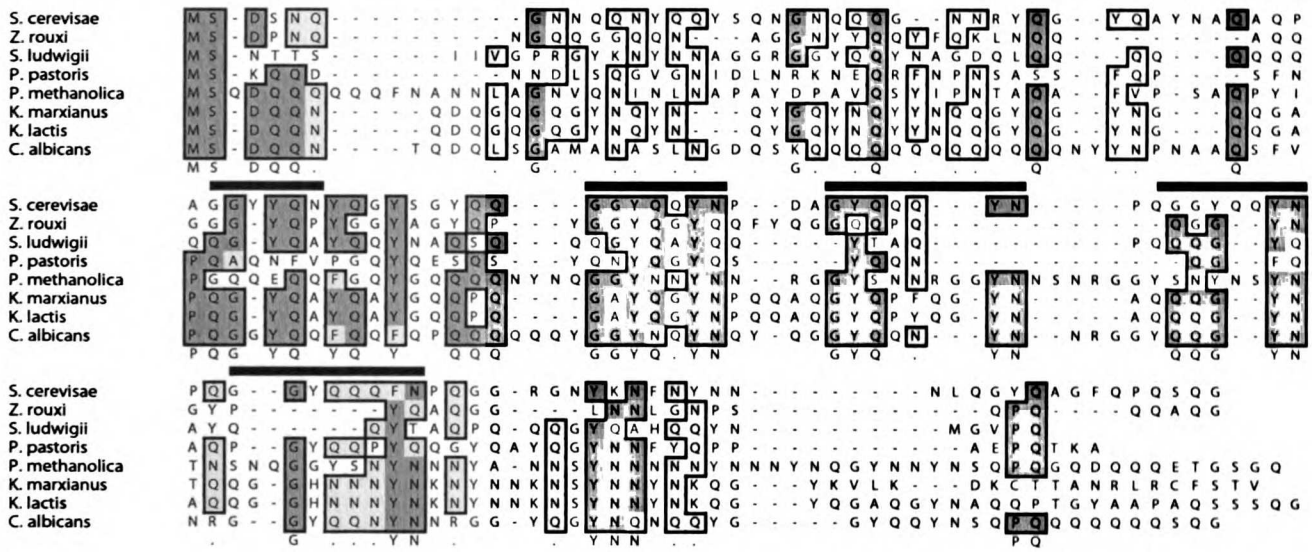

S. cerevisae
Z. rouxi
S. ludwigii
P. pastoris
P. methanolica
K. marxianus
K. lactis
C. albicans

Figure 1. Evolutionary Analysis of Sup35p PrDs

(A) Phylogenetic relationship of yeast species based on 26S RNA sequences (Kurtzman, 1994). For comparison, the fission yeast *S. pombe* whose Sup35p does not have a PrD (Ito et al., 1998), is shown. Scale (percent divergence) is denoted on top. Resende et al. have previously submitted a full length sequence of *SUP35* from *C. albicans* (AF020554) to public databases.

(B) Plot of percent of charged (Arg, Lys, Asp, Glu) versus Gln/Asn residues for the Sup35 PrD homologs as well as the *S. cerevisiae* Ure2p prion domain. For comparison, the full-length *S. cerevisiae* ORFs including specifically the entire Sup35p and Ure2p proteins are also shown.

(C) Amino acid sequence comparison of PrD homologs. Amino acid identities and similarities are indicated by dark gray and light gray boxes, respectively. Sequences were aligned using the ClustalW algorithm (Higgins et al., 1996). The black bar denotes the approximate location of the oligopeptide repeats.

adenine-free media. As expected, when *S. cerevisiae* PrDM ($PrDM_{SC}$) was used in the fusion protein, [PSI^+] yeast retained the suppression phenotype (Figure 2A). In contrast, expression of fusion proteins containing PrDMs from *C. albicans*, *K. lactis* and *P. methanolica*, denoted $PrDM_{CA}$, $PrDM_{KL}$, and $PrDM_{PM}$ respectively, conferred antisuppression to [PSI^+] cells, suggesting that they are not inactivated by the endogenous *S. cerevisiae* aggregate.

In the visual assay, the in vivo aggregation state is observed directly using an inducible PrDM fused to GFP (Patino et al., 1996). As expected, upon $PrDM_{SC}$-GFP induction, punctate foci appear rapidly in the majority of [PSI^+] cells (Figures 2B and C). In contrast, [psi^-] cells show a prolonged diffuse cytoplasmic fluorescence, with foci forming slowly. Expression of the fusion of the N-terminal regions of *C. albicans*, *K. lactis* and *P. methanolica* to GFP also resulted in formation of foci. Indeed, de novo formation of foreign PrDM foci in [psi^-] cells, especially of $PrDM_{PM}$-GFP, is faster than that of *S. cerevisiae* PrDM. In contrast to $PrDM_{SC}$-GFP, however, the in vivo kinetics of foreign PrDM aggregation are similar in [psi^-] and [PSI^+] cells (Figure 2C). Together, these assays show that although the ability of these PrDs to aggregate is conserved, the foreign prion domains are not incorporated into the endogenous Sup35p aggregate present in [PSI^+] yeast.

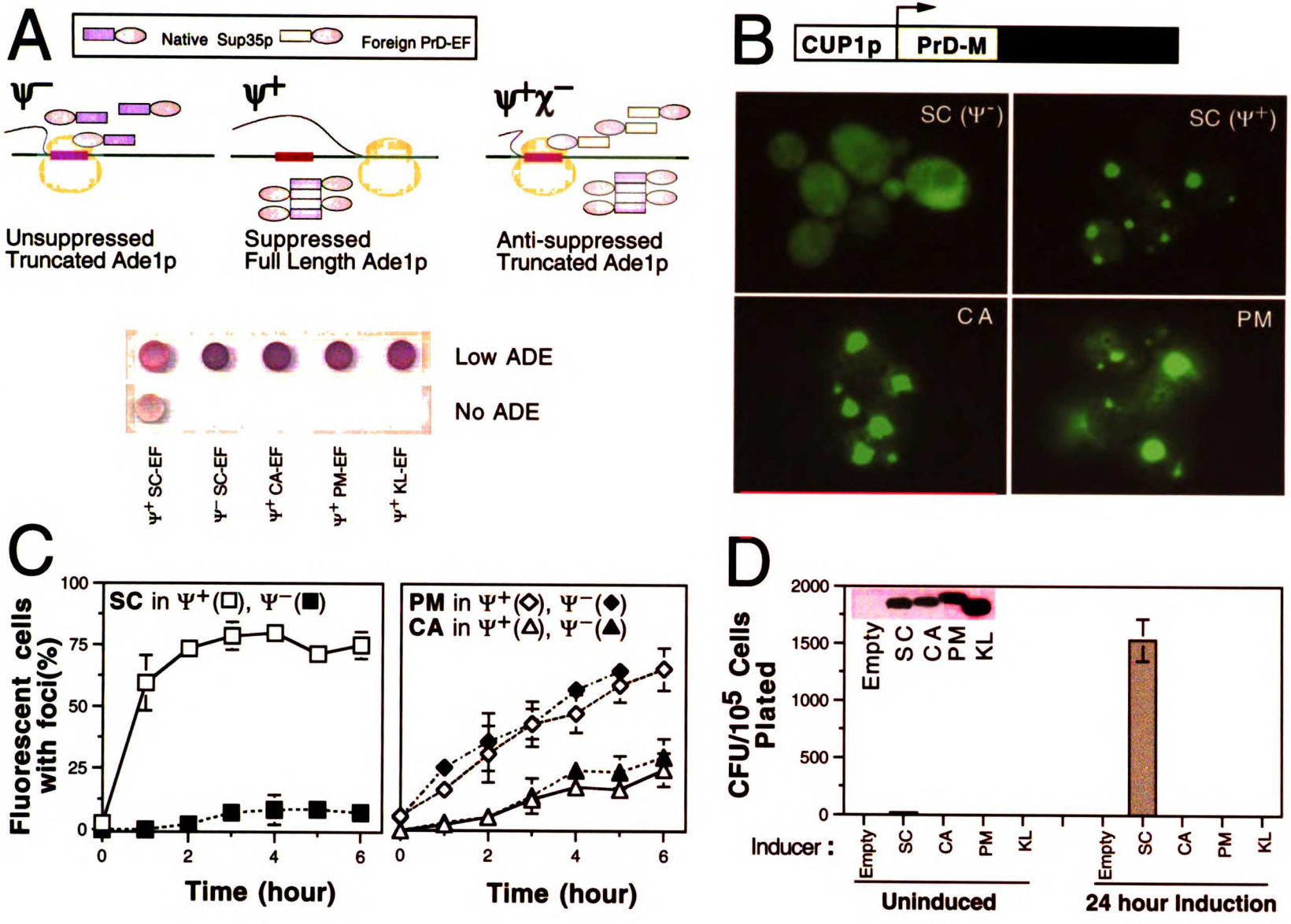

A
Native Sup35p
Foreign PrD-EF
Ψ⁻
Ψ⁺
Ψ⁺χ⁻
Unsuppressed Truncated Ade1p
Suppressed Full Length Ade1p
Anti-suppressed Truncated Ade1p
Low ADE
No ADE
Ψ⁺ SC-EF
Ψ⁻ SC-EF
Ψ⁺ CA-EF
Ψ⁺ PM-EF
Ψ⁺ KL-EF
B
CUP1p
PrD-M
SC (Ψ⁻)
SC (Ψ⁺)
CA
PM
C
Fluorescent cells with foci(%)
SC in Ψ⁺(□), Ψ⁻(■)
PM in Ψ⁺(◇), Ψ⁻(◆)
CA in Ψ⁺(△), Ψ⁻(▲)
Time (hour)
D
CFU/10⁵ Cells Plated
Empty
SC
CA
PM
KL
Inducer :
Uninduced
24 hour Induction

Figure 2. Examination of the Ability of Foreign PrDs to Interact with *S. cerevisiae* PrD

Throughout the figures CA, KL, PM, and SC refer to $PrDM_{CA}$, $PrDM_{KL}$, $PrDM_{PM}$, and $PrDM_{SC}$, respectively. Y^+ and Y^- indicate the presence or absence of the *S. cerevisiae* PrD aggregate. C^+ and C^- indicate the presence or absence of the foreign PrDM aggregates. All quantitative experiments were conducted in triplicate and errors are indicated.

(A) Effect of foreign PrDM-EF fusion proteins on the [PSI^+] suppression phenotype. [PSI^+] yeast expressing the indicated species of PrDM-EF fusion were plated on low or no ADE medium. For comparison, [psi-] yeast are also shown. On top is shown a schematic model explaining the antisuppression phenotype (red colonies on low ADE and lack of growth on no ADE) resulting from expression of foreign PrDM. In [psi^-] cells, soluble Sup35p mediates translational termination at the ade1 nonsense mutation (red bar). In suppressed [PSI^+] yeast, absence of soluble Sup35p results in translational readthrough. Failure of foreign PrDM-EF to be incorporated into the [PSI^+] amyloid leads to soluble EF and antisuppression.

(B) Illustration of GFP visual assay. On top is shown a schematic of the copper inducible PrDM-GFP plasmid. From left to right are

examples of diffuse GFP fluorescence in [psi$^-$] cells, discrete foci in [PSI$^+$] cells and de novo aggregates formed by overexpression of $PrDM_{CA}$-GFP, and $PrDM_{PM}$-GFP as observed by fluorescence microscopy.

(C) Quantitative foci formation kinetics. [PSI$^+$] and [psi$^-$] yeast, as indicated, containing either $PrDM_{SC}$-GFP (left) or the indicated foreign PrDM-GFP (right) were induced in early-log phase, and results are plotted as the percentage of fluorescent cells with visible foci as a function of induction time.

(D) Induction of [PSI$^+$] by overexpression of PrDM-GFP. [psi$^-$] yeast containing a plasmid encoding the indicated species of PrDM-GFP or an empty control plasmid were plated on media lacking adenine either before (uninduced) or after 24 hours of copper induction. The number of [PSI$^+$] colonies (CFUs) per 10^5 cells is plotted. (Inset) Immunoblots of inducer PrDM expression following induction.

Conversely, we asked if foreign PrD aggregates could incorporate soluble *S. cerevisiae* Sup35p present in [psi$^-$] yeast. Aggregates formed by transient overexpression of PrDM$_{SC}$, either by itself or fused to GFP, incorporate native full length Sup35p, leading to a permanent conversion to [PSI$^+$] (Chernoff et al., 1993; Patino et al., 1996). After 24 hours of induction, ~1.5% of [psi$^-$] cells convert to [PSI$^+$] (Figure 2D). In contrast, similar levels of overexpression of foreign PrDM-GFPs, even at timepoints when foci are readily observable, fail to induce [PSI$^+$] conversion (Figure 2D and inset). Thus the foreign PrD aggregates are unable to seed the in vivo aggregation of *S. cerevisiae* Sup35p.

Hereafter, we designate the aggregation state of the foreign PrDs by [CHI]. For example, in [psi$^-$ CHI$^+$] yeast, endogenous Sup35p is soluble and the foreign PrD is aggregated, whereas in [psi$^-$ chi$^-$] yeast both the endogenous and foreign Sup35p are soluble.

Foreign PrDs Form Stable Prions that are Limited by a Species Barrier

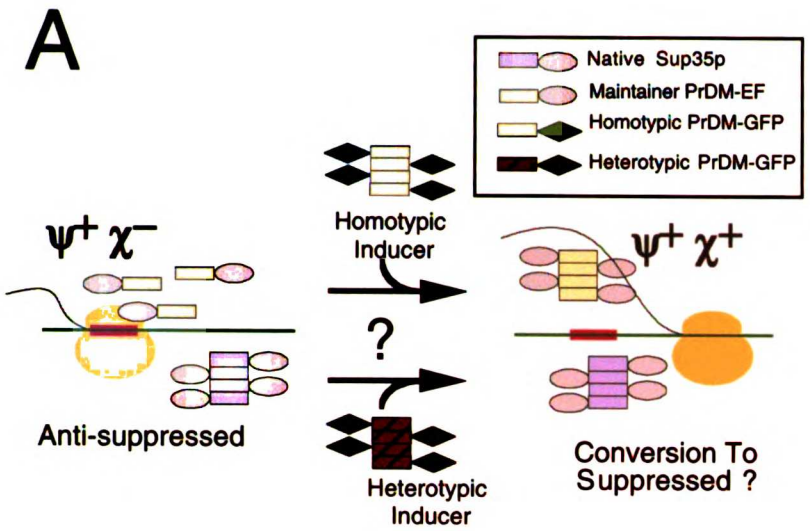
Given the long evolutionary distance separating these yeast species, the failure of the foreign PrDs to interact with *S. cerevisiae* prions is not surprising, but leaves unresolved the question of

whether the foreign PrDs behave as prions. To address this question, we devised a novel genetic system which allowed us to monitor the induction and propagation of [CHI$^+$] (Figure 3A). Here, two plasmids were introduced into yeast. The first, termed the maintainer plasmid, encodes an epitope-tagged PrDM fused to *S. cerevisiae* EF domain, termed PrDM-EF, under control of the *S. cerevisiae SUP35* promoter. The second inducer plasmid encodes a PrDM-GFP fusion protein under control of the inducible *CUP1* promoter. Transient overexpression of the inducer protein, either from the same (homotypic) or different (heterotypic) species as the maintainer PrD, results in de novo formation of the GFP fusion aggregates, thus mimicking infection experiments used in studies of mammalian prions. The ability of the inducer aggregates to "infect" the maintainer PrD is monitored by the permanent change in suppression phenotype.

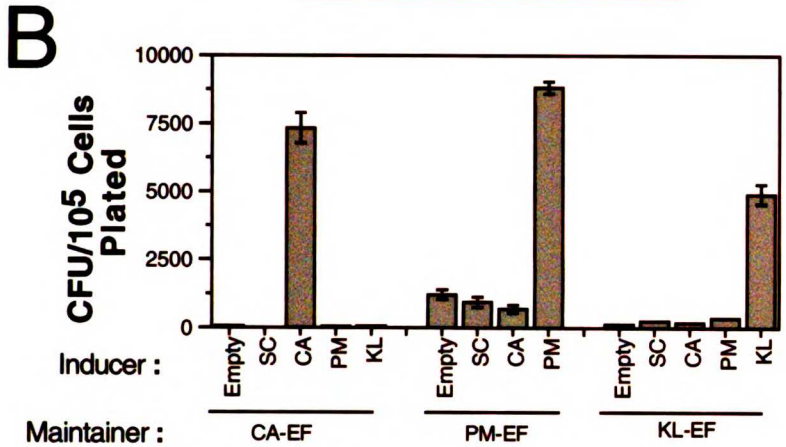
This system allowed us to test whether the foreign PrD aggregates behave as prions, and if so, whether divergence in sequences leads to a species barrier to prion propagation (Figure 3A). In support of the species barrier model, we found that the conversion of the maintainer PrD is induced only upon overexpression of the homotypic inducer PrD. For example, overexpression of $PrDM_{CA}$-GFP in the presence of $PrDM_{CA}$-EF

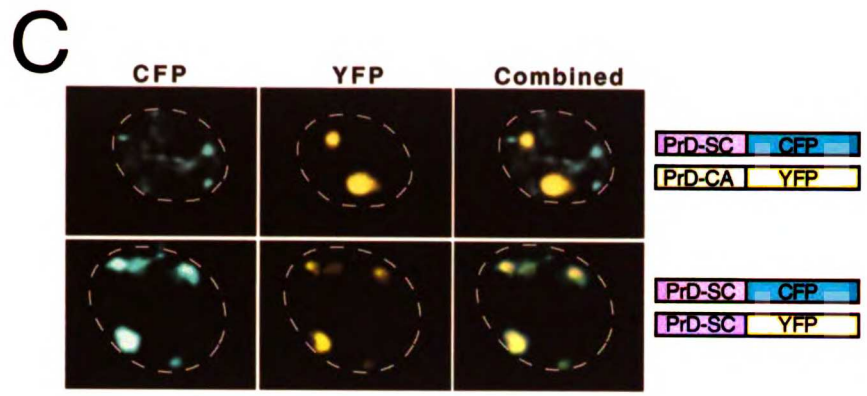
maintainer, resulted in ~7.5% adenine-prototrophic [PSI$^+$ CHI$_{CA}^+$] colonies (Figure 3B). By contrast, less than 0.005% [chi$_{CA}^-$] cells were converted when an empty control plasmid or any of the heterotypic inducers were used. Likewise, in the presence of PrDM$_{KL}$-EF and PrDM$_{PM}$-EF maintainers, only the overexpression of the homotypic inducers caused efficient conversion to the [CHI$^+$] state. Finally, double fluorescence experiments using fusions between PrDM and two color variants of GFP showed that prion aggregates from two different PrD species do not co-localize (Figure 3C).

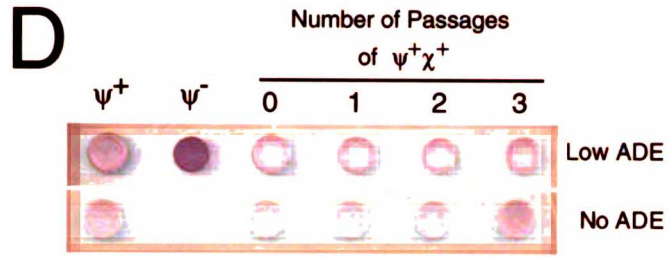
We next asked whether the [CHI$^+$] prion state could propagate stably. [PSI$^+$ CHI$^+$] yeast were sequentially patched onto medium which selects for the maintainer plasmid, but neither the inducer plasmid nor the prion state. Interestingly, some [PSI$^+$ CHI$^+$] isolates rapidly reverted back to unsuppressed [chi$^-$] state, whereas other strains propagated the [CHI$^+$] aggregate robustly. After three successive patches, corresponding to ~60 generations, a typical strong [PSI$^+$ CHI$_{CA}^+$] strain had lost the inducer plasmid but could still grew on medium lacking adenine. Even more remarkably, no red sectors were observed on low adenine media, demonstrating that even in the absence of selection yeast uniformly retained the [CHI$^+$] prion (Figure 3D).

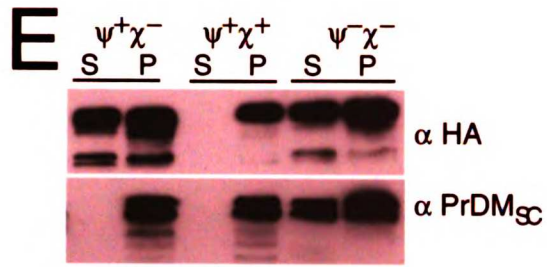


	Homotypic Inducer	Heterotypic Inducer
Not Prion	No Conversion	No Conversion
Species Barrier	Conversion	No Conversion
No Barrier	Conversion	Conversion









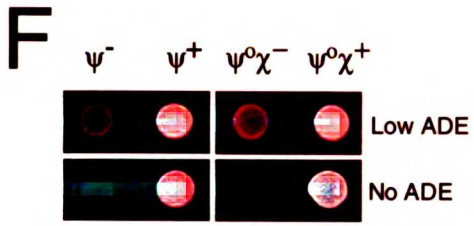


Figure 3. Examination of the Ability of Homotypic or Heterotypic PrD Overexpression to Induce [CHI$^+$] Prions

(A) Schematic of [CHI$^+$] two-plasmid induction assay. Expression of foreign PrDM-EF from the maintainer plasmid in [PSI$^+$ chi$^-$] cells results in soluble EF activity, leading to antisuppression. Aggregates are introduced by transient overexpression of the PrDM-GFP fusion either from the same (homotypic) or different (heterotypic) species as the maintainer. The ability of the introduced aggregates to convert the PrDM-EF fusion yielding [PSI$^+$ CHI$^+$] yeast can be monitored by a persistent change in suppression phenotype. Below, for each of the three indicated models, the predicted effect of homotypic and heterotypic inducers on [CHI$^+$] conversion is shown.

(B) Induction of [CHI$^+$] by overexpression of homotypic and heterotypic PrDM-GFP inducers. [PSI$^+$ chi$^-$] yeast containing HA epitope-tagged *C. albicans*, *P. methanolica*, or *K. lactis* maintainer plasmid indicated by CA-EF, PM-EF, and KL-EF, respectively, and the indicated inducer plasmid were grown in selective medium to early-log phase. Following 40 hours of copper induction, the number of [PSI$^+$ CHI$^+$] colonies (CFUs) was determined by plating onto no ADE medium.

(C) In vivo observation of species specific aggregation. Shown are fluorescence images of [PSI$^+$] yeast co-expressing fusion proteins between the indicated species of PrDM and a yellow (YFP) and cyan (CFP) variant of GFP driven by an inducible copper promoter. The left, middle and right panels display the Cyan, Yellow and Combined fluorescence, respectively. Schematics on the right indicate the identity of the PrDM-GFP variant fusion proteins. Dotted lines denote the cell outlines.

(D) Stability of propagation of a [CHI$^+$] prion. A robust [PSI$^+$ CHI$_{CA}$$^+$] isolate was patched serially onto complete medium. Following the indicated number of passages, an aliquot was patched on either low or no ADE medium, as indicated, to test for the presence of the suppression phenotype caused by [CHI$^+$]. For comparison, [psi$^-$] and [PSI$^+$] yeast are shown.

(E) Centrifugation assay to examine the solubility of foreign PrDM-EF. Extracts from the indicated yeast strains were centrifuged at 100,000g, and soluble (S) or pelleted (P) fractions were assayed by immunoblots with antibodies (a-HA) specific to the epitope-tagged PrDM$_{CA}$-EF or with antibodies (α PrDM$_{SC}$) specific to the *S. cerevisiae* Sup35p.

(F) Formation and propagation of [CHI$^+$] in the absence of *S. cerevisiae* Sup35p. A yeast strain was constructed in which the

chromosomal *SUP35* gene was deleted and replaced with an episomal copy $PrDM_{CA}$-EF. Shown are examples of this strain either prior to or after induction of the [CHI^+] prion, denoted [Y° C^-] and [Y° C^+], respectively, plated on either low or no ADE medium. For comparison [PSI^+] and [psi^-] yeast are also shown.

The reversible loss of the [PSI$^+$] factor, by overexpression of the molecular chaperone HSP104 or by exposure to guanidine hydrochloride, provided critical evidence that [PSI$^+$] inheritance is mediated by a change in protein conformation rather than by a DNA element (Chernoff et al., 1995; Wickner, 1994). HSP104 overexpression and guanidine caused solubilization of the [CHI$_{CA}^+$] aggregate, suggesting that both [CHI$^+$] and [PSI$^+$] prions are cured by similar mechanisms (Figure 3E and data not shown). Furthermore, the [CHI$_{CA}^+$] prion can propagate even in yeast lacking the endogenous sup35 gene (Figure 3F). As with [PSI$^+$ CHI$^+$] yeast, transient exposure to guanidine also cures the [CHI$^+$] state. Together, these data provide strong genetic evidence that foreign PrDs can support prion-based inheritance and that a barrier prevents cross-seeding between different species of PrDs.

Using a centrifugation assay (Patino et al., 1996; Paushkin et al., 1996), we confirmed biochemically that the [CHI$^+$] suppression phenotype results from a heritable aggregation of the foreign PrDM-EF protein. Aggregated Sup35p from [PSI$^+$] yeast extract fractionates to the pellet following high-speed centrifugation, whereas soluble Sup35p from [psi$^-$] yeast extract remains largely in the supernatant. When subjected to this centrifugation assay,

$PrDM_{CA}$-EF protein from [CHI_{CA}^{+}] yeast fractionates to the pellet (Figure 3E). By contrast, it remains largely in the supernatant both in the unconverted and the guanidine or HSP104 cured [chi^{-}] strains (Figure 3E, and data not shown).

Selective Seeding of Fibril Formation Recapitulates the Species Barrier In Vitro

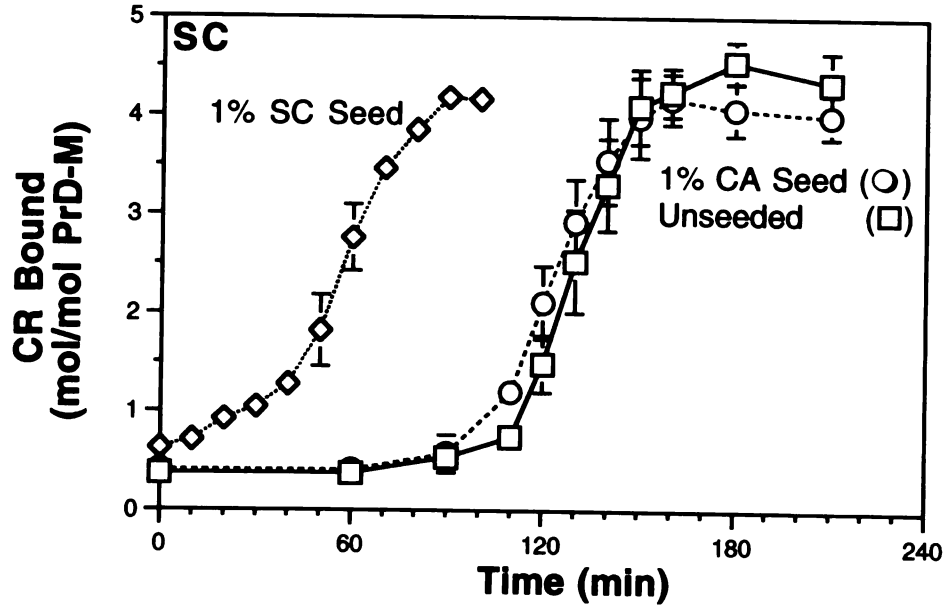
Purified Sup35p forms self-seeding amyloids in vitro, thereby providing a simple biochemical system to examine the molecular basis of the observed species barrier (DePace et al., 1998; Glover et al., 1997; King et al., 1997; Paushkin et al., 1997). After dilution of purified $PrDM_{SC}$ from denaturant, there is an initial lag phase of ~120 minutes, followed by a cooperative conversion from random coil to β-sheet rich amyloid fibrils. This conversion can be readily monitored by selective binding of the fibrils to the dye Congo red (Figure 4A). Importantly, the addition of pre-formed fibril catalyzes this conversion by eliminating the lag phase, thus recapitulating in vitro the self-propagation of the prion state.

We attempted to recapitulate [CHI^{+}] prion propagation in vitro using purified *C. albicans* PrDM proteins ($PrDM_{CA}$). Upon dilution from denaturant, $PrDM_{CA}$ exhibited a cooperative transition to fibrils

following a lag phase of ~140 minutes. As with $PrDM_{SC}$, addition of a small amount (1%) of pre-formed $PrDM_{CA}$ fibrils eliminated the lag phase (Figure 4B). Strikingly, the addition of $PrDM_{CA}$ fibrils, even at amounts which could efficiently catalyze $PrDM_{CA}$ amyloid formation, did not convert $PrDM_{SC}$ (Figure 4A). Conversely, addition of $PrDM_{SC}$ seed did not alter the kinetics of $PrDM_{CA}$ fibril formation (Figure 4B). Thus our in vitro seeding experiments mirror a key aspect of the in vivo homotypic conversion experiments, in which prion formation is initiated only by homotypic PrD overexpression.

In the selective seeding experiments above, addition of a fibril seed failed to convert PrDM from another species. However, for yeast to stably exist in a [PSI^+ chi^-] state, the foreign PrD must remain soluble in the presence of continuous aggregation of similar levels of the endogenous Sup35p. To better simulate this condition, we tested the effect of selective seeding of a solution containing an equal concentration of $PrDM_{SC}$ and $PrDM_{CA}$ monomers. Addition of a small amount of $PrDM_{SC}$ seed (5%) shortly after the initiation of the polymerization reaction caused an immediate conversion detected by Congo red binding (Figure 5A). Consistent with the notion that ongoing $PrDM_{SC}$ polymerization does not induce conversion of $PrDM_{CA}$, the curve plateaus at a level corresponding to conversion of half of the total protein. Subsequent addition of $PrDM_{CA}$ seed (5%)

A


SC
1% SC Seed
1% CA Seed (O)
Unseeded (□)
CR Bound
(mol/mol PrD-M)
Time (min)
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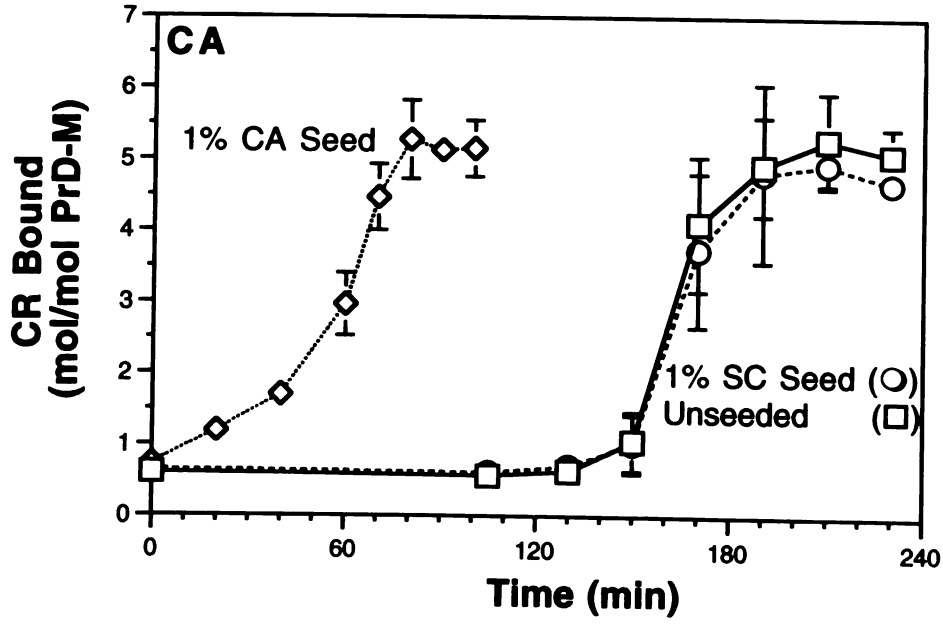
1
2
3
4
5
0
60
120
180
240

B


CA
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CR Bound
(mol/mol PrD-M)
Time (min)
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1
2
3
4
5
6
7
0
60
120
180
240

Figure 4. In Vitro Amyloid Fibril Formation of $PrDM_{SC}$ and $PrDM_{CA}$

To initiate conversion, concentrated pure PrDM protein in urea was diluted into conversion buffer and subjected to continuous slow rotation. At indicated times, the extent of fibril formation was assayed by Congo red binding. Each curve was conducted in triplicate. Bars indicate errors larger than the symbol size.

(A) Conversion kinetics of 2.5 μM *S. cerevisiae* PrDM in the absence (square), and presence of 1% (wt/wt) pre-formed $PrDM_{SC}$ (diamond) and $PrDM_{CA}$ (circle) fibrils.

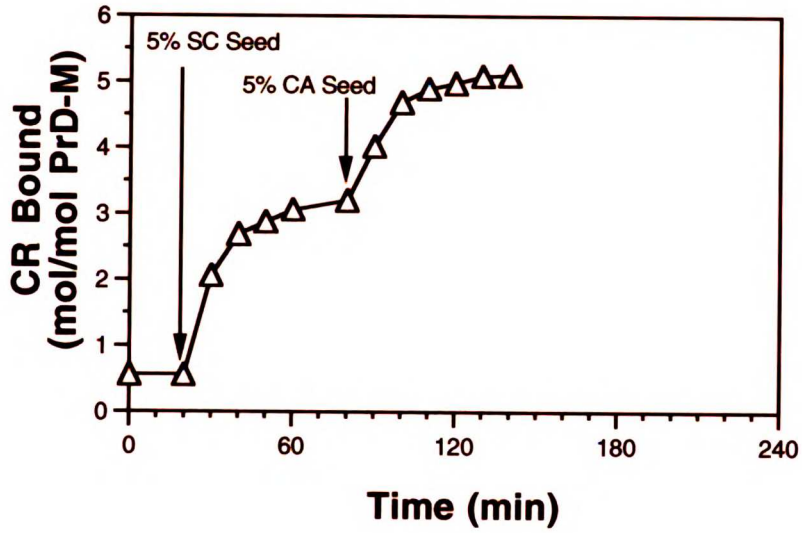
(B) Conversion kinetics of 2.5 μM *C. albicans* PrDM in the absence (square), and presence of 1% (wt/wt) pre-formed $PrDM_{CA}$ (diamond) and $PrDM_{SC}$ (circle) fibrils.

initiated the conversion of the remaining protein. In contrast, if no seed or more $PrDM_{SC}$ seed was added, there was no immediate conversion. Instead a slow rise in Congo red binding was observed with kinetics indistinguishable from that of the spontaneous conversion of $PrDM_{CA}$ (Figure 5B). Immuno-electron microscopy confirmed that these two prions had a strong preference to form amyloid fibrils composed of a single species. We used a species-specific polyclonal antibody to label *S. cerevisiae* PrDM with 5-nm gold particles and a monoclonal epitope-tag specific antibody to label $PrDM_{CA}$ with 15-nm gold. In a reaction where equimolar amounts of $PrDM_{SC}$ and $PrDM_{CA}$ were polymerized simultaneously, individual fibrils were labeled with only one size of gold particles, suggesting that, as observed in vivo in the double fluorescence studies (Figure 3C), fibrils were composed exclusively of a single species of PrD (Figure 5C).

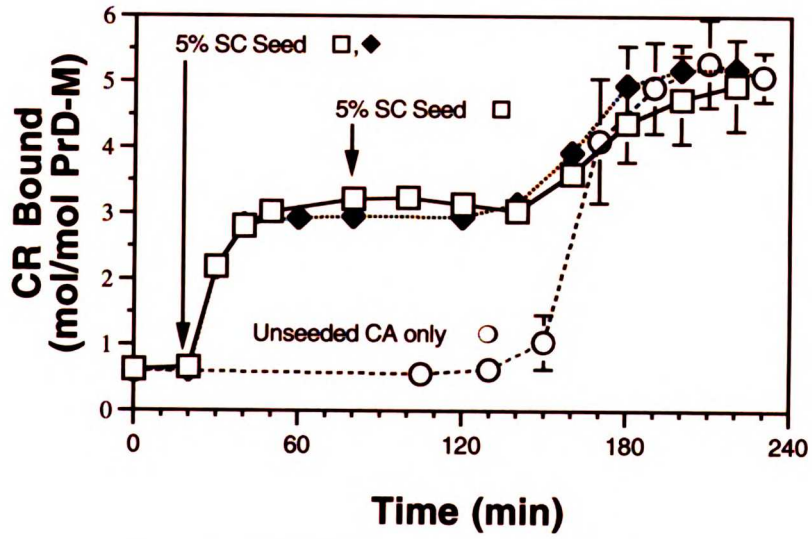
Prion Specificity is Encoded in a Short Region of the PrD Domain

Taking advantage of the visual and [PSI$^+$] conversion assays described above, we next asked whether a specific region of the PrD is responsible for the species barrier. We created two complementary chimeric proteins, the first of which ($PrDM_{SC1\text{-}39CA}$)

A


5% SC Seed
5% CA Seed
CR Bound
(mol/mol PrD-M)
Time (min)
0
1
2
3
4
5
6
0
60
120
180
240

B


5% SC Seed □,◆
5% SC Seed □
Unseeded CA only ○
CR Bound
(mol/mol PrD-M)
Time (min)
0
1
2
3
4
5
6
0
60
120
180
240

C

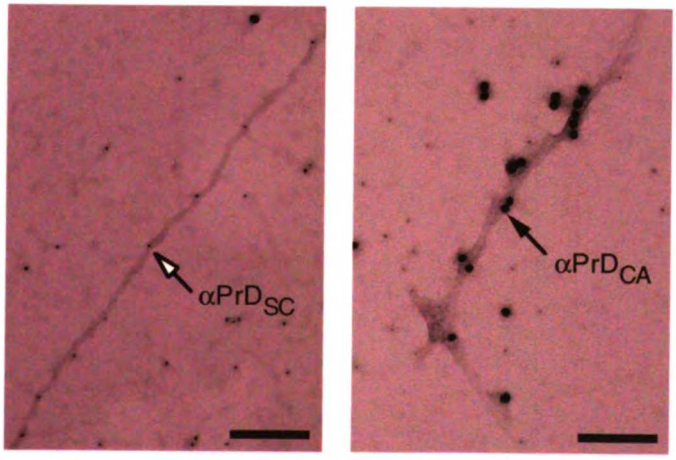

αPrD$_{SC}$
αPrD$_{CA}$

Figure 5. In vitro Amyloid Fibril Formation of a $PrDM_{SC}$ / $PrDM_{CA}$ Mixture

(A) Conversion kinetics of equimolar mixture (2.5μM) of $PrDM_{SC}$ and $PrDM_{CA}$ seeded with 5% (wt/wt) $PrDM_{SC}$ fibril and subsequently with $PrDM_{CA}$ fibril at indicated timepoints.

(B) Conversion kinetics of an equimolar mixture (2.5μM) of $PrDM_{SC}$ and $PrDM_{CA}$ seeded with 5% (wt/wt) $PrDM_{SC}$ fibril only (filled diamond) twice with $PrDM_{SC}$ fibril (square) compared to unseeded kinetics of $PrDM_{CA}$ (circle).

(C) Electron micrographs of the converted $PrDM_{SC}$ and $PrDM_{CA}$ mixture. $PrDM_{SC}$ and $PrDM_{CA}$ are labeled by 5nm and 15nm gold particles, respectively, using species specific antibodies. Examples of fibers decorated exclusively with 5nm (left) or 15nm (right) gold particles from the same electron micrograph are shown. Although some fibrils were poorly labeled, no fibers were decorated by both antibodies. Scale bar corresponds to 100nm.

contained residues 1 to 39 from *S. cerevisiae* with the remaining PrD sequence from *C. albicans*, and the second ($PrDM_{CA1-39SC}$), in which the first 39 residues of $PrDM_{SC}$ were replaced with the *C. albicans* sequence (Figure 6A). Microscopic examination of GFP fusions shows that both chimeras form aggregates (Figure 6C and data not shown). However, while over-expression of $PrDM_{SC1-39CA}$ was highly effective at converting [psi$^-$] yeast to [PSI$^+$], over-expression of $PrDM_{CA1-39SC}$ had no detectable effect (Figure 6B).

A previous study had found that mutations which cause poor incorporation into or curing of wild type [PSI$^+$] aggregates cluster to a short region composed of residues 8 to 26 (DePace et al., 1998). To test whether this epitope is sufficient to allow crossing of the species barrier, we constructed a third chimera consisting of residues 8 to 26 of *S. cerevisiae* replacing the corresponding *C. albicans* sequence, denoted $PrD_{SC8-26CA}$ (Figure 6A). This chimeric PrDM-GFP retains the ability to aggregate and is seeded by the endogenous Sup35p aggregate, as shown by the visual assay (Figure 6C). More remarkably, it induces conversion of [psi$^-$] yeast to [PSI$^+$] with only a modestly reduced efficiency (~ 2 fold) compared with wildtype $PrDM_{SC}$ (Figure 6B). These data indicate that a short peptide epitope composed of residues 8 to 26, presumably on the growing face of the amyloid, is sufficient to mediate specificity in the

incorporation of monomers into the polymerizing fibril (Figure 6D, see Discussion).

Identification of a Novel Prion-forming Protein in *S. cerevisiae*

The observation that all known yeast prion proteins have high Gln/Asn content and few charged residues (Figure 1B) suggests that proteins with similar properties could form prions. A search of genomic databases revealed that several other proteins have domains with similar properties (Figure 7A and M. Michelitsch and J.S.W., in preparation). We experimentally examined the ability of one such domain from a protein encoded by the uncharacterized ORF *YPL226W* (*NEW1*) to form a prion using the two-plasmid assay described above. We fused the first 153 amino acids of New1p to an HA_3-epitope-tagged EF domain and expressed this fusion protein, termed New1$p_{1\text{-}153}$-HA_3-EF, driven by the *SUP35* promoter. Initially, this fusion protein complemented the deficiency of Sup35p activity associated with [PSI$^+$], indicating that it is not inactivated by the [PSI$^+$] prion; we termed this antisuppressed state [PSI$^+$ nu$^-$]. However, transient over-expression of the same fragment fused to GFP, but not over-expression of $PrDM_{SC}$-GFP or $PrDM_{CA}$-GFP, caused ~10 percent of [PSI$^+$ nu$^-$] cells to convert to a suppressed state termed [PSI$^+$ NU$^+$] (Figure 7B). Similarly to [CHI$^+$], we found

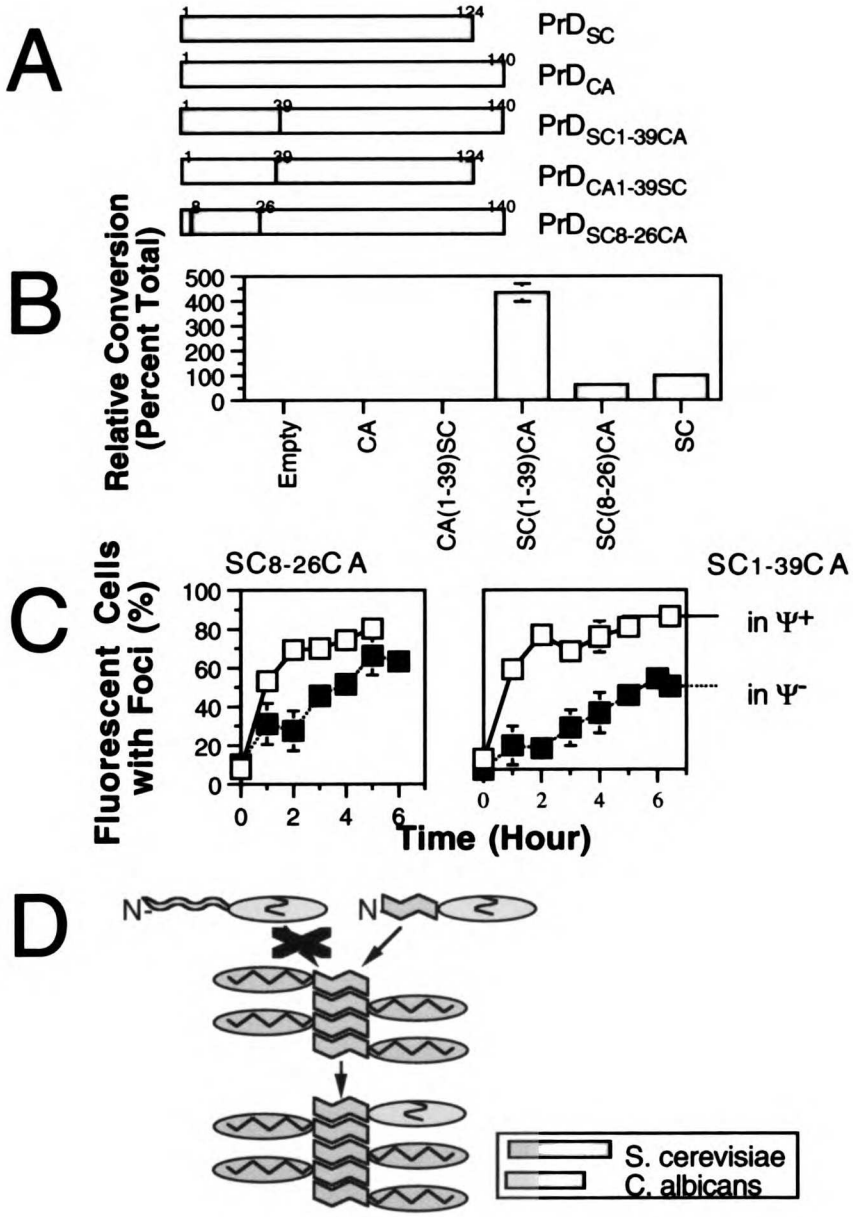

A
PrD$_{SC}$
PrD$_{CA}$
PrD$_{SC1-39CA}$
PrD$_{CA1-39SC}$
PrD$_{SC8-26CA}$
B
Relative Conversion (Percent Total)
Empty
CA
CA(1-39)SC
SC(1-39)CA
SC(8-26)CA
SC
C
Fluorescent Cells with Foci (%)
SC8-26CA
SC1-39CA
in Ψ+
in Ψ-
Time (Hour)
D
N
S. cerevisiae
C. albicans

Figure 6. Chimeric Analysis of PrD

(A) Schematics of chimeric PrD sequences. Residue numbers are indicated on top. Chimeric region residue numbers are that of *S. cerevisiae* sequence. *S cerevisiae* regions are denoted by purple and *C. albicans* by light yellow.

(B) Efficiency, relative to $PrDM_{SC}$, of conversion of [psi$^-$] to [PSI$^+$] by overexpression of the indicated PrDM-GFP fusion.

(C) Quantitative foci formation kinetics. [PSI$^+$] and [psi$^-$] yeast, as indicated, containing the indicated chimeric PrDM-GFP fusion were grown in selective medium and induced at early-log phase. Shown is a plot of the percentage of fluorescent cells with visible foci as a function of induction time.

(D) Hypothetical model to explain chimera conversion data. The N-terminal region of PrD is envisioned as having many of the critical species-specific interactions involved in recruitment of new monomers to the growing amyloid, whereas the C-terminal region may be primarily involved in intramolecular interactions that stabilize the prion form.

significant variation among [NU$^+$] isolates, with some rapidly reverting to [nu$^-$] and others propagating stably (data not shown). Finally, centrifugation analysis directly demonstrated that the [NU$^+$] state results from aggregation of the New1p-EF fusion (Figure 7C). Taken together, these observations argue the Gln/Asn-rich N-terminal region of New1p can support a prion mechanism of inheritance.

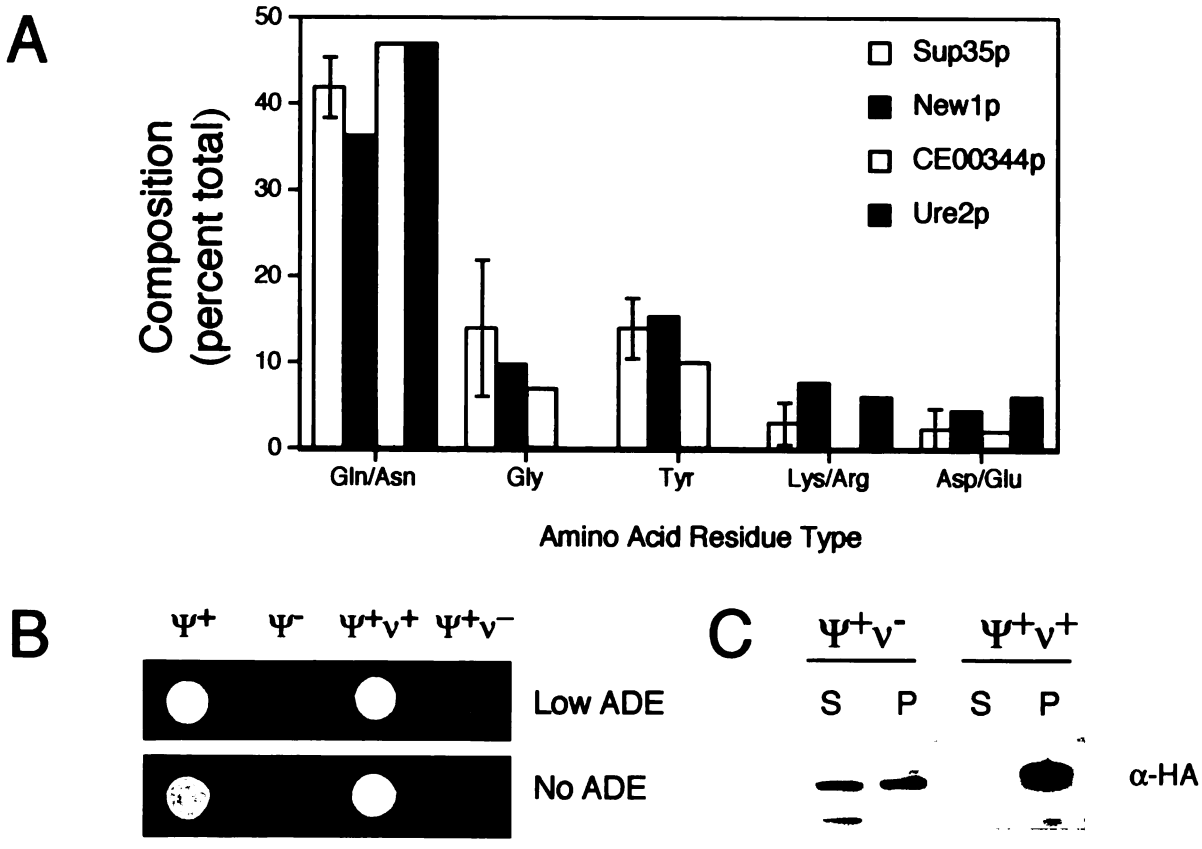

A
Composition (percent total)
50
40
30
20
10
0
Sup35p
New1p
CE00344p
Ure2p
Gln/Asn
Gly
Tyr
Lys/Arg
Asp/Glu
Amino Acid Residue Type
B
Ψ+
Ψ-
Ψ+ν+
Ψ+ν−
Low ADE
No ADE
C
Ψ+ν-
Ψ+ν+
S
P
S
P
α-HA

Figure 7. The N-terminus of New1p Can Act as a Prion.

(A) The amino acids content of Sup35p PrD, residues 10-100 of New1p, residues 230 to 319 from the *C. elegans* ORF CE00344, and the first 90 amino acids of Ure2p are displayed. For the PrD plot, the average value among the various yeast species is used with error bars indicating the maximum variation.

(B) Phenotypic consequences of prion formation by the New1p$_{1-153}$-HA$_3$-EF fusion protein. Shown are examples of [PSI$^+$ nu$^-$] and [PSI$^+$ NU$^+$] isolates grown on medium with low amounts of adenine (above) and no adenine (below), illustrating the conversion from an antisuppressed to a suppressed state following [Nu$^+$] induction. For comparison, [PSI$^+$] and [psi$^-$] strains are shown.

(C) Centrifugation assay to follow the solubility of the New1p$_{1-153}$-HA$_3$-EF fusion, performed as in Figure 3E.

Discussion

To investigate how prions can specifically propagate in the complex cellular milieu, we have cloned and characterized the N-terminal prion domain (PrD) from a range of budding yeasts. Despite the long evolutionary distances separating these species (Kurtzman, 1994), the Sup35p homologs examined contain PrDs capable of forming prions. In particular, upon induction of aggregates by overexpression, the foreign PrDs switch from an initially soluble [chi$^-$] state to an aggregated [CHI$^+$] prion state. This prion state can stably propagate until cured by guanidine treatment or HSP104 over-expression. As with mammalian prions (Prusiner et al., 1998), a species barrier prevents prion aggregates from one species from converting soluble PrDs of another.

Surprisingly, even in identical genetic backgrounds, different [CHI$^+$] isolates of the same foreign PrD species as well as different [NU$^+$] isolates show markedly different stability and levels of nonsense suppression. Analogous strain differences were found previously in [PSI$^+$] as well as in mammalian prions (Derkatch et al., 1996; Prusiner et al., 1998; Zhou et al., 1999). Initially discovered as differences in pathology between isolates of scrapie, mammalian prion strains were thought to result from nucleic acid variations,

arguing against a protein-mediated mechanism of prion infectivity. It now appears that the presence of stable, distinct strains, possibly arising from different prion conformations, is an inherent property of prions.

Insights into the Molecular Architecture of the Prion Domain: Specificity and Stability Domains Within the PrD

The mammalian prion species barrier, which prevents the spread of scrapie and bovine spongiform encephalopathy to man, has been the focus of intense research efforts (Prusiner et al., 1998). Many questions about the molecular basis of the prion species barrier remain. For example, in vitro experiments indicate that specific interactions between PrP^{SC} and PrP^{C} lead to a species barrier in generating the protease-resistant form (Horiuchi and Caughey, 1999; Kocisko et al., 1995). In vivo chimera analyses, however, suggest that species-dependent interactions between the prion particles and an unidentified host factor, termed Protein X, also contribute substantially to the species barrier (Telling et al., 1995). These issues have been difficult to resolve because of the inability thus far either to produce de novo infectious PrP^{SC} or to effectively recapitulate sustained prion propagation in vitro.

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Our data show that for the yeast [PSI$^+$] prion, the species barrier results from a remarkable specificity in interaction between the prion protein itself, mediated by a well-defined epitope in the PrD (Figure 6D). First, for all of the species examined, only overexpression of homotypic PrD induces prion formation. Second, as with [PSI$^+$] (Chernoff et al., 1995; Tuite et al., 1981), transient overexpression of the molecular chaperone HSP104 or exposure to guanidine, which most likely acts by modulating cellular factors, cures the [CHI$_{CA}^+$] prion. Thus, at least for these components, the ability of the *S. cerevisiae* folding machinery to regulate the aggregation state of the *C. albicans* PrD is conserved. Third, in vitro selective seeding experiments faithfully recapitulate the species barrier. Strikingly, even in an equimolar solution of PrDM$_{SC}$ and PrDM$_{CA}$, seeded polymerization of the *S. cerevisiae* protein leads to rapid formation of pure PrDM$_{SC}$ fibrils without affecting the polymerization kinetics of the *C. albicans* protein. Finally, chimeric analyses reveal that prion specificity of *S. cerevisiae* can be conferred to the PrD of another species by a substitution of 19 amino acid residues near the PrD N-terminus.

This localization of a species-determining region helps reconcile disparate results from previous efforts to dissect PrD function. A screen for mutant PrDs that either fail to interact with or

cause curing of the endogenous [PSI$^+$] prion found that these mutations located between residues 8 and 26 (DePace et al., 1998). However, sequences C-terminal to this region have been shown to be critical for prion function (Doel et al., 1994; Liu and Lindquist, 1999). Moreover, expansion of the imperfect oligopeptide repeats, also located outside of this species-determining domain, dramatically enhances the rate of prion formation. These observations, together with our in vitro selective seeding experiments, suggest a model (Figure 6D) in which the N-terminus resides on the surface of the growing amyloid and contributes much of the specificity of PrD monomer recruitment. By contrast, the more C-terminal region might largely be involved in intra-molecular interactions that stabilize the prion form. Substitution of *S. cerevisiae* residues 8-26 into PrD_{CA} would thus change the surface of the amyloid, allowing it to incorporate PrD_{SC} monomers. By contrast, expansion or deletion of the imperfect oligopeptide repeats would lead to increased or decreased stabilization of the PrD, thereby modulating its tendency to form amyloids without altering prion specificity.

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An Epigenetic Switch: Prion-based Protein Regulation?

Alleles of the mammalian prion protein prone to prion formation are rare, as would be expected given the devastating effects of these diseases. By contrast, the ability of the N-terminal domain of Sup35p to form a prion is conserved across the budding yeasts. This functional conservation is remarkable since the PrD sequence is not strongly conserved and earlier mutational analyses showed that even single point mutations in the *S. cerevisiae* PrD can inhibit prion formation (DePace et al., 1998). These observations raise the intriguing possibility that rather than being pathogenic, [PSI$^+$] might be an evolutionarily beneficial state. However, we can not rule out the possibility that the retention of prion function is a by-product of conservation of an unidentified PrD function (Bailleul et al., 1999). Consistent with a beneficial role for the [PSI$^+$] prion, Tuite and coworkers found that following exposure to high temperature or ethanol some [PSI$^+$] yeasts show enhanced survival compared to isogenic [psi$^-$] yeasts (Eaglestone et al., 1999). In addition, we find that over-expression of PrDM$_{CA}$-GFP in *C. albicans* induces formation of punctate foci, although it remains to be seen whether these aggregates can propagate in a prion-like manner (unpublished observations).

As a mechanism of inheritance, prions provide a number of potentially advantageous features (Lindquist, 1997). Prion formation allows a cell to inhibit the activity of a specific protein and propagate this state indefinitely while retaining the potential to restore the original protein activity. Moreover, the rate of conversion to and from the prion state can be dramatically enhanced by changes in the environment (Tuite et al., 1981). Finally, because prion domains are modular (Patino et al., 1996; Ter-Avanesyan et al., 1993), fusion to prion domains could potentially allow prion-based regulation of a broad range of proteins.

For a prion to serve as an epigenetic switch, it must propagate specifically without interfering with other proteins. Specificity of prion interactions, resulting from differences in primary sequence and manifested as a barrier to cross-species prion induction, could serve as mechanism to prevent such improper interactions. Consistent with the proposal that multiple prion states could propagate independently in the same cell, we have identified a new prion-forming domain in *S. cerevisiae* (the N-terminal portion of New1p) and have shown that its prion state propagates independently of [PSI$^+$]. Furthermore, we find that over-expression of a Ure2p fragment which efficiently induces the [URE3] prion (Edskes et al., 1999) does not induce [PSI$^+$] nor is Ure2p

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incorporated into [PSI$^+$] aggregates (data not shown), indicating that the existence of one prion within a cell does not promote the appearance of others.

If multiple different prions can exist independently within a single cell, then how many different prions are there? The spectrum of Sup35p PrD sequences, together with earlier mutational analyses, provide a wealth of data to search for novel prions. Despite little strict sequence conservation, all of the examined Sup35p PrDs as well as the Ure2p prion domain contain an extremely high Gln/Asn and low charge content (Figures 1B and 7A). Moreover, in some neurodegenerative diseases, expansion of polyGln repeats leads to intranuclear aggregates in vivo and self-propagating amyloids in vitro (Bates et al., 1998). A search of genomic databases for domains with amino acid content comparable to Sup35p PrDs revealed a handful of such domains in both yeast and nematodes (Figure 7A and M. Michelitsch and JSW, in preparation), at least one of which (New1p) forms a prion in yeast. The challenge now is to determine how many other Gln/Asn rich domains can form stable, self-propagating prions, and what the physiological role for such novel prions may be.

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Experimental Procedures

General Procedures and Reagents

Isogenic *S. cerevisiae* [psi$^-$] and [PSI$^+$] strains 74-D694 [*Mat* **a**, *ade1-14*(UGA), *his3, leu2, trp1, ura3*] (Chernoff et al., 1995) were used for all experiments except the Sup35p deletion studies which used YJW541 [*Mat* **a**, *ade1-14, his3, leu2, trp1, ura3, sup35::TRP1*]. *C. albicans* SC5314, *K. marxianus* and *P. pastoris* were gifts from A. Johnson, E. Blackburn and C. Craik, respectively. *K. lactis* and *S. ludwigii* were gifts from I. Herskowitz. *P. methanolica* (56509) and *Z. rouxi* (48232) were obtained from ATCC. Nucleic acid, immunoblot and yeast manipulations were performed according to standard protocols (Ausubel, 1987). All plasmid sequences were confirmed by dye termination sequencing (Perkin-Elmer).

Oligonucleotide primers were as follows:
P1 GGGGGATCCGTCGACACTAGTACAATGTCTGACCAACAGAATACT;
P2 CCCAGATCTTCTAGAATCCTTGACAACTTCTTCGTC;
P3 GGGGGATCCGTCGACACTAGTACAATGTCTCAAGATCAACAGCAA;
P4 CCCAGATCTTCTAGAATCGTTGACAATGGAGGCATC;
P5
CGACGAGGATCCGTCGACATGTCAGACCAACAAAATCAAGACCAAGGG;
P6 CAAAGTGAATTCAGATCTATCTTTAACGACTTCTTC;
P7 GGGCGGCATATGTCTGACCAACAGAATACTCAG;
P8 GCCCGAATTCTTAGTGATGATGGTGATGGTGGTGATCCTTGACAACTTC

TTCGTC;

P9
CCGGAATTCTTAATGGTGATGATGGTGATGAGCGTAATCTGGAACGTCAT
A;
P10 AACGGTTGGGTCATCCATCTT;
P11 TTTGTTGGTATCCATGACCCATGACAAGTACCA;

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P12
GGCCCAGTGAGCAGAGTGACGGAGGACTCGAGCTCAAGCTAATCCGGC
GTGCA TTGAC;
P13 GATCGTCAATGCACGCCGGATTTACGCC;
P14 CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
P15 ACCTGCCC;
P16 GCGCGTCGACATGCCTCCAAAGAAGTTTAAGG;
P17
GCGCCGAATTCGGGAGATCTTTGATTTTTGCAATCAGTGATACTTT
GACA TTCAGG;
Q0 CCAGTGAGCAGAGTGACG;
Q1 GACTCGAGCTCAAGCTAA;
AP-1 CCATCCTAATACGACTCACTATAGGGC;
AP-2 ACTCACTATAGGGCTCGAGCGGC.

Cloning of foreign SUP35p PrDs

To clone Sup35 PrDM domains, we modified the RACE procedure for capturing cDNA 5' ends (Frohman, 1993) to allow analysis of genomic DNA. Yeast genomic DNA from the desired species was digested to completion by a restriction enzyme that results in either 5' GATC overhangs or blunt ends. Linkers compatible with either the GATC (annealed P12, P13) or blunt ends (annealed P14, P15) were then ligated onto the ends of the genomic fragments. PCR amplification was performed using the ligated fragments as templates, the EF specific primer P10 and linker primer Q0 or AP1 for the GATC or blunt ended fragments, respectively. Amplified products were used as templates in a second round of PCR using nested EF specific primer P11 and linker primer Q1 or AP2 for the GATC and blunt ended derived fragments, respectively. For

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reactions resulting in a single distinct band, PCR products were purified by agarose gel electrophoresis and sequenced.

Plasmid Construction

All yeast expression vectors used a previously described (DePace et al., 1998) modular insert composed of a promoter domain flanked by 5' Xho1 and 3' BamHI sites, a PrDM/New1p1-153 domain flanked by 5' BamHI/Sal1 and 3'BglII/EcoRI sites, and an EF/GFP/eCFP/eYFP domain flanked by 5'EcoRI and 3'SacI sites. For the maintainer plasmid, the insert was cloned into the XhoI-SacI sites of a URA3 marked CEN/ARS plasmid (pRS316), and a triple HA epitope was inserted between the BglII/EcoRI sites. For the inducer plasmids, the insert was cloned into the XhoI-SacI sites of a LEU2 marked 2μm plasmid (pRS425). To create the foreign PrDM inducer and maintainer plasmids, $PrDM_{CA}$ (primers P1, P2), $PrDM_{PM}$ (P3, P4), or $PrDM_{KL}$ (P5, P6) domains were PCR amplified from genomic DNA and inserted into the BamHI/EcoRI sites of the appropriate $PrDM_{SC}$ encoding plasmid. To create the New1p inducer and maintainer plasmids, the first 153 codons of *NEW1* were PCR amplified (P16 and P17) and inserted into the Sal1/EcoR1 sites of the appropriate $PrDM_{SC}$ encoding plasmid. The chimeras $PrDM_{SC1\text{-}39CA}$ (encoding a protein in which residues 40-124 of $PrDM_{SC}$ were replaced with

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residue 44-140 from $PrDM_{CA}$), $PrDM_{CA1-39SC}$ (encoding a protein in which residue 1-39 of $PrDM_{SC}$ was replaced with residue 1-44 of $PrDM_{CA}$) and $PrDM_{SC8-26CA}$ (encoding a protein in which residue 1- 7 and 27-124 of $PrDM_{SC}$ were replaced with residues 1-7 and 30-140 from $PrDM_{CA}$, respectively) were all derived from the $PrDM_{SC}$ inducer plasmid by seamless cloning (Stratagene). Plasmids encoding eCFP and eYFP were obtained from Clontech. For bacterial expression, 6xHis tagged $PrDM_{CA}$ with (P7, P9) or without (P7, P8) an HA tag was PCR amplified and inserted into NdeI/EcoRI sites of a T7 expression vector.

In Vivo GFP Foci Formation

Yeast carrying the indicated species of PrDM-GFP inducer plasmid were grown to early-log phase in SD-LEU, and induced with 50 μM $CuSO_4$. At indicated times, cells were examined by fluorescent microscopy (Olympus BX60) and photographed by CCD camera (Photometrics). For quantitative measurement, random fields were chosen and percentage of fluorescent cells with punctate foci were calculated. Double fluorescence images were collected by wide-field 3D deconvolution microscopy (Agard et al., 1989) using filters optimized for CFP and YFP fluorescence (Chroma).

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[PSI$^+$] Conversion Assay and Two-Plasmid Assay for Aggregation

For [PSI$^+$] conversion assay, [psi$^-$] yeast freshly transformed with the indicated inducer plasmids were grown in 10ml SD-LEU to early-log phase and induced with 50 μM $CuSO_4$. After 24 hours, samples were plated onto SD-ADE. For the two-plasmid [CHI$^+$] conversion assay, [PSI$^+$] yeast freshly transformed with the indicated species of maintainer and inducer plasmids were grown in 10 ml SD-URA-LEU to early-log phase and induced with 50 μM $CuSO_4$. At indicated times, samples were plated onto SD-URA-ADE. For both assays, after five days incubation at 30°C, visible colonies were counted. For the [CHI$^+$] induction experiments in the sup35 deletion strain, YJW541 initially carrying p316SpSupEF (Depace et al, 1998) and a HIS marked PrDMCA-EF maintainer plasmid were grown on 5-FOA to ensure loss of p316SpSupEF yielding the [psi° chi$^-$] strain. The [psi° CHI$^+$] was derived by transient overexpression of $PrDM_{CA}$ from an inducer plasmid, selection on medium lacking ADE and subsequent loss of inducer plasmid. For the [NU$^+$] induction experiments, [PSI$^+$] yeast freshly transformed with the New1p inducer and maintainer plasmids and treated as described above for the [CHI$^+$] experiments.

Immuno-Electron Microscopy

PrDM fibrils (8μg) produced from a conversion reaction containing equimolar $PrDM_{SC}$ and $PrDM\text{-}HA_{CA}$ were incubated with 50μg rabbit polyclonal antibody raised against $PrDM_{SC}$ (HTI Bio-products) and 100μg of the mouse monoclonal anti-HA antibody 16B12 (Babco) for 20 minutes at room temperature. 5 μl each of 5 nm gold conjugated to anti-rabbit and 15 nm gold conjugated anti-mouse secondary antibodies (Nanoprobes) were added and incubated for additional 20 minutes. The solution was added to a glow discharged carbon coated nickel grid, washed extensively with water, and stained with 2% uranyl acetate. Electron micrographs were collected with EM400 Transmission Electron Microscope (Phillips).

Other Assays

Centrifugation assays were performed as previously described (DePace et al., 1998). Recombinant PrDM was purified under denaturing conditions as described previously (DePace et al., 1998; Glover et al., 1997). Congo red binding assays were carried out as described previously (DePace et al., 1998; Klunk et al., 1989).

Acknowledgments

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Chapter III. Multiple Gln/Asn-rich Prion Domains Confer Susceptibility to Induction of the Yeast *[PSI$^+$]* Prion

Summary

The yeast prion *[PSI$^+$]* results from self-propagating aggregates of Sup35p. *De novo* formation of *[PSI$^+$]* requires an additional non-Mendelian trait, thought to result from a prion form of one or more unknown proteins. We find that the Gln/Asn-rich prion domains of two proteins, New1p and Rnq1p, can control susceptibility to *[PSI$^+$]* induction as well as enhance aggregation of a human glutamine expansion disease protein. *[PSI$^+$]* inducibility results from gain-of-function properties of New1p and Rnq1p aggregates rather than from inactivation of the normal proteins. These studies suggest a molecular basis for the epigenetic control of *[PSI$^+$]* inducibility and may reveal a broader role for this phenomenon in the physiology of protein aggregation.

Introduction

Infectious, self-propagating protein aggregates (prions) constitute a conformational mechanism of inheritance. Originally described as mammalian pathogens (Prusiner, 1998), prions have recently been shown to underlie a number of non-Mendelian traits in fungi (Wickner et al., 1999). While the prion proteins responsible for these various phenomena are otherwise unrelated, a common feature of prion conversion is the formation of b-sheet-rich, amyloid aggegrates. In mammals, prions cause neurodegeneration and death, but in *Saccharomyces cerevisiae* they allow the epigenetic control of protein activity, which can in certain circumstances prove adaptive (Eaglestone et al., 1999; True and Lindquist, 2000).

The best-characterized yeast prions are *[URE3]* and *[PSI$^+$]*, which result from the aggregation of the nitrogen catabolism repressor Ure2p and the translation termination factor Sup35p, respectively. Incorporation of these proteins into prion aggregates diminishes their normal activity, emulating Mendelian loss-of-function mutations; for example, *[PSI$^+$]* causes the suppression of certain nonsense mutations. The prion properties of Ure2p and Sup35p depend on glutamine- and asparagine-rich (Gln/Asn-rich)

domains at the amino terminus of each protein (Serio and Lindquist, 1999; Wickner et al., 1999). These prion domains are dispensable for the normal functions of their proteins and are modular, conferring the ability to aggregate when transferred to other proteins (Wickner et al., 2000).

Genomic searches for Gln/Asn-rich domains similar to the Sup35p prion domain have lead to the identification of prion domains in two previously uncharacterized proteins, New1p and Rnq1p (Michelitsch and Weissman, 2000; Santoso et al., 2000; Sondheimer and Lindquist, 2000). When the Gln/Asn-rich regions of these proteins were fused to the C-terminal translational termination domain of Sup35p, the resulting fusion proteins could reversibly aggregate to form stably heritable prion states, termed *[NU$^+$]* and *[RPS$^+$]*, which emulated the *[PSI$^+$]* nonsense suppression phenotype. Full-length Rnq1p exists predominantly in an aggregated form in a number of laboratory strains, but this prion state causes no apparent change in phenotype (Sondheimer and Lindquist, 2000).

Glutamine-rich aggregates are also associated with human disease (Perutz, 1999). Several heritable neurodegenerative diseases including Huntington's Disease and a variety of spinocerebellar ataxias are caused by expansion of CAG codons leading to the production of proteins with long polyglutamine

(polyGln) tracts. In vivo, these aberrant proteins form inclusions in affected neurons that correlate with neurodegeneration. Furthermore, expanded polyGln tracts within the Huntingon's Disease protein cause it to form self-seeding amyloid fibrils in vitro (Scherzinger et al., 1999). The structural basis of Gln/Asn-rich aggregation is thought to involve formation of "polar zippers" in which the b-sheets are stabilized by a network of hydrogen bonds involving glutamine and aspargine sidechains (Perutz et al., 1994).

Although Gln/Asn-rich domains have an inherent propensity to form amyloid aggregates in vitro, cellular factors strongly modulate the formation, propagation and toxicity of such aggregates in vivo. Notably, the propagation of *[PSI$^+$]* in yeast as well as of polyGln aggregates in yeast, worms and mammalian cells can be modulated by Hsp104p, a yeast chaperone involved in thermal tolerance and disaggregation of misfolded proteins (Carmichael et al., 2000; Chernoff et al., 1995; Krobitsch and Lindquist, 2000; Lindquist et al., 1995; Satyal et al., 2000). Elevated levels of Hsp70 chaperones can also ameliorate the effect of polyGln protein aggregation and can influence the efficiency of *[PSI$^+$]* propagation in yeast [Warrick, 1999 #7;Newnam, 1999 #46;Chernoff, 1999 #44;Satyal, 2000 #2;Muchowski, 2000 #15;Kobayashi, 2000 #16].

In addition to genome-encoded factors such as chaperones, a remarkable additional element, which is itself inherited in a non-Mendelian manner, regulates the *de novo* appearance of *[PSI$^+$]* (Derkatch et al., 2000; Derkatch et al., 1997). In yeast strains possessing the *[PSI$^+$]* inducibility factor (termed *[PIN$^+$]*), transient over-expression of the Sup35p prion domain leads to the appearance of *[PSI$^+$]* at a high frequency. In contrast, strains lacking the *[PIN$^+$]* factor (referred to as *[pin$^-$]*) do not convert to *[PSI$^+$]* upon Sup35p over-expression. Propagation of *[PIN$^+$]* does not depend on Sup35p and, like known yeast prions, *[PIN$^+$]* is inherited in a cytoplasmic manner, requires the presence of Hsp104p and can be eliminated by treatment of cells with guanidine. Despite these arguments for a prion basis for *[PIN$^+$]*, the specific prion-forming protein or proteins associated with the *[PIN$^+$]* state have remained mysterious.

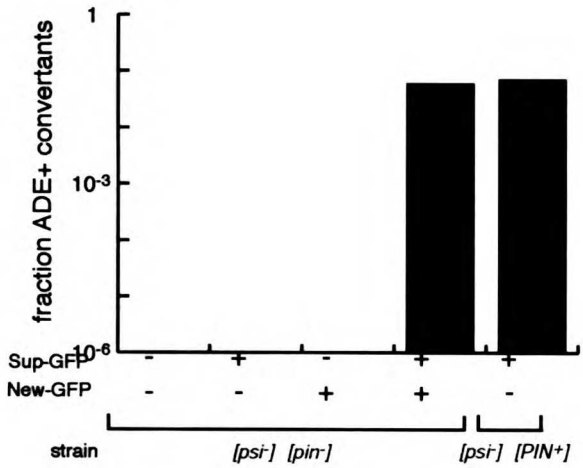
We thus have two new prion proteins with unknown functions (New1p and Rnq1p) and an epigenetic prion-like phenomenon (*[PIN$^+$]*) without a known protein. Here we establish that prion forms of both New1p and Rnq1p can promote *[PSI$^+$]* formation. The identification of *[PSI$^+$]*-inducing prions has allowed us to examine the mechanism of prion formation and to uncover a broader role for prions in modulating polyGln aggregation.

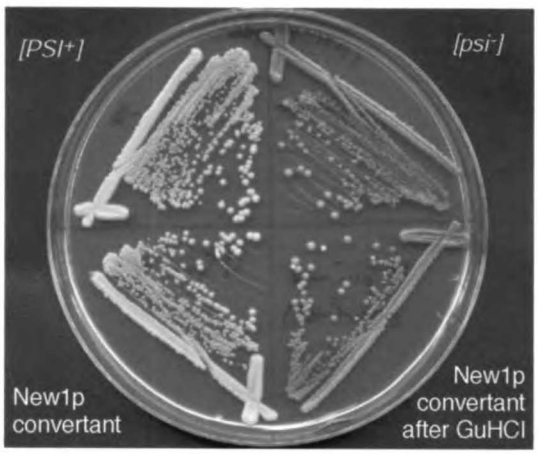
Results

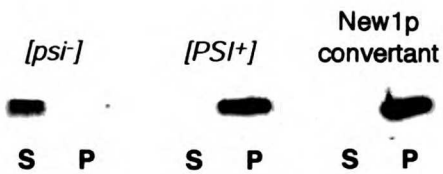
Over-expression of non-Sup35p prion domains confers *[PSI⁺]* susceptibility

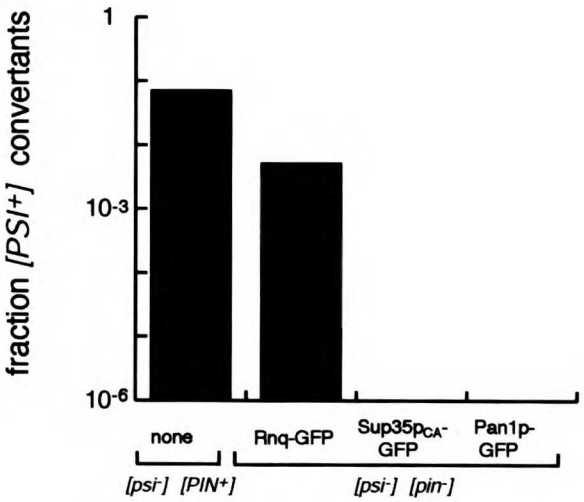
To test whether newly identified prions could, like *[PIN⁺]*, act as *[PSI⁺]*-promoting factors, we examined the effect of New1p aggregates on *[PSI⁺]* induction. For these studies, we transiently over-expressed fusion proteins composed of either the New1p prion domain (residues 1-153) or the Sup35p prion domain (residues 1-253) and the green fluorescent protein, termed New–GFP and Sup-GFP, respectively. In contrast to Sup-GFP, which does not aggregate in *[pin⁻]* strains, over-expressed New-GFP formed visible aggregates (see Figure 4D). We assayed for *[PSI⁺]* induction by using strains with a nonsense mutation in the *ADE1* gene (*ade1-14*) that permits growth on medium lacking adenine (-ade) as a result of *[PSI⁺]*-mediated nonsense suppression.

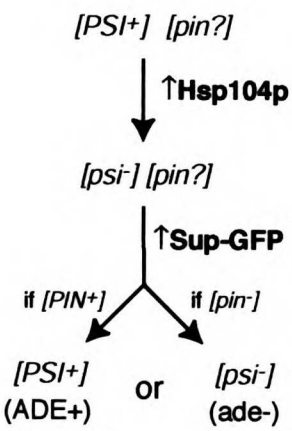
We found that over-expression of New-GFP circumvented the requirement for *[PIN⁺]* in *[PSI⁺]* induction. As expected, over-expression of New-GFP or Sup-GFP alone did not induce *[PSI⁺]* in *[pin⁻]* strains (conversion frequencies $<10^{-6}$, Figure 1A). However, over-expression of both Sup-GFP and New-GFP in a *[pin⁻]* strain caused the appearance of adenine prototrophic (ADE+) colonies at a frequency ($\sim 6 \times 10^{-2}$) comparable to that occurring after over-


A
fraction ADE+ convertants
1
10-3
10-6
Sup-GFP
New-GFP
strain
[psi-] [pin-]
[psi-] [PIN+]


B
[PSI+]
[psi-]
New1p convertant
New1p convertant after GuHCl


C
[psi-]
[PSI+]
New1p convertant
S P
S P
S P


D
fraction [PSI+] convertants
1
10-3
10-6
none
Rnq-GFP
Sup35pCA-GFP
Pan1p-GFP
[psi-] [PIN+]
[psi-] [pin-]


E
[PSI+] [pin?]
↑Hsp104p
[psi-] [pin?]
↑Sup-GFP
if [PIN+]
if [pin-]
[PSI+] (ADE+)
or
[psi-] (ade-)

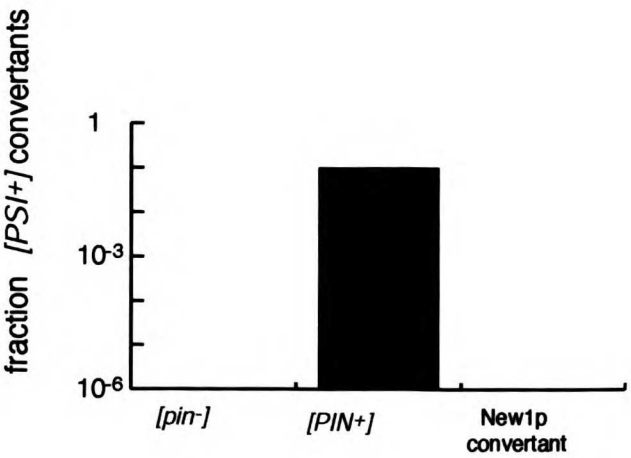

F
fraction [PSI+] convertants
1
10-3
10-6
[pin-]
[PIN+]
New1p convertant

Figure 1. New1p and Rnq1p prion domains facilitate conversion to *[PSI$^+$]*

(A) New-GFP over-expression circumvents the requirement for *[PIN$^+$]* in *[PSI$^+$]* induction. The indicated proteins were over-expressed in a *[psi$^-$] [pin$^-$]* strain, and the frequency of conversion to ADE+ was determined by plating equal quantities of cells onto media with or without adenine. For comparison, a *[psi$^-$] [PIN$^+$]* control expressing Sup-GFP alone is shown. In these and subsequent prion induction experiments, each column represents the sum of 2-5 independent experiments in which 200-400 colonies were counted.

(B) Reversibility of ADE+ in New1p-mediated convertants. ADE+ convertants obtained by dual over-expression of Sup-GFP and New-GFP (New1p convertant) are shown before and after treatment with 5 mM guanidine hydrochloride (GuHCl) along with *[PSI$^+$]* and *[psi-]* controls.

(C) Biochemical evidence that New1p-mediated convertants are *[PSI$^+$]*. Extracts from the indicated strains were subjected to ultra-centrifugation; the presence of Sup35p in the supernatant (S) or pellet (P) was determined by immunoblotting after SDS-PAGE.

(D) Effect of other Gln/Asn-rich domains on *[PSI$^+$]* induction. Residues 153-415 of Rnq1p, 1-295 of Sup35p$_{CA}$ and 1-240 of Pan1p

were fused to GFP and tested for *[PSI⁺]* induction when over-expressed in a *[psi⁻] [pin⁻]* strain together with Sup-GFP. Sup-GFP alone in a *[psi⁻] [PIN⁺]* strain is shown at left. In all strains, over-expression of these proteins alone did not induce *[PSI⁺]* (data not shown). The lower efficiency of *[PSI⁺]* induction by Rnq-GFP compared to New-GFP is likely due to the lower propensity of Rnq-GFP to aggregate *de novo* in *[pin⁻]* strains.

(E) Scheme for ascertaining the presence of *[PIN⁺]* in New1p- and Rnq1p-mediated *[PSI⁺]* convertants. The *[PSI⁺]* factor is eliminated by over-expression of Hsp104p, then Sup-GFP is transiently over-expressed. If the strain is *[PIN⁺]*, *[PSI⁺]* should reappear (bottom left), whereas in *[pin⁻]* strains Sup-GFP over-expression should not induce *[PSI⁺]* (bottom right).

(F) New1p-mediated *[PSI⁺]* convertants are *[pin⁻]*. We used the scheme in (E) to test for the presence of *[PIN⁺]* in a New1p-mediated convertant. For comparison, conversions rates in *[pin⁻]* and *[PIN⁺]* strains are shown.

expression of Sup-GFP alone in a *[PIN⁺]* strain. We obtained similar results using full length New1p in place of New-GFP (data not shown).

Several observations argued that these ADE+ convertants resulted from the *de novo* appearance of the *[PSI⁺]* prion. First, they formed visible colonies after ~5 days on -ade and displayed variable degrees of strength and stability of the ADE+ trait, features typical of fresh *[PSI⁺]* inductants (Derkatch et al., 1996). Additionally, the ADE+ property of these convertants could be abolished by treatment with guanidine (Tuite et al., 1981), which cures all known yeast prions (Figure 1B). Finally, in extracts prepared from a New1p-converted strain, Sup35p fractionated entirely to the pellet following ultra-centrifugation (Figure 1C), indicating that the ADE+ property of these strains arose from prion formation by Sup35p (Patino et al., 1996; Paushkin et al., 1996).

We tested whether other Gln/Asn-rich proteins could promote *[PSI⁺]* induction by over-expressing GFP fusions of the prion domains of two other known prion-forming proteins, Rnq1p and *Candida albicans* Sup35p ($Sup35p_{CA}$) (Santoso et al., 2000; Sondheimer and Lindquist, 2000), as well as the Gln/Asn-rich portion of Pan1p. We saw varying levels of GFP aggregation in *[pin⁻]* strains expressing each of these proteins alone. However, only the

Rnq-GFP fusion promoted *[PSI⁺]* induction, although at a lower frequency (5×10^{-3}) than did New-GFP (Figure 1D). Thus, only a subset of Gln/Asn-rich aggregates facilitate *[PSI⁺]* induction.

The induction of *[PSI⁺]* by dual over-expression of Sup-GFP and New-GFP could result from the conversion of the cells from *[pin⁻]* to *[PIN⁺]*, which would then permit *[PSI⁺]* induction by excess Sup-GFP (Derkatch et al., 2000). However, when we eliminated the *[PSI⁺]* factor from the New-GFP- or Rnq-GFP-mediated inductants using Hsp104p over-expression (a treatment which does not cure *[PIN⁺]* (Derkatch et al., 1997)), the resulting *[psi⁻]* strains proved refractory to re-induction of *[PSI⁺]* by over-expression of Sup-GFP alone (Figures 1E,F and data not shown), indicating that these strains had remained *[pin⁻]*. Thus, New1p- and Rnq1p-mediated conversion from *[psi⁻]* to *[PSI⁺]* occurred without conversion to *[PIN⁺]*.

[PSI⁺] susceptibility does not arise from inactivation of chromosomally-encoded New1p and Rnq1p proteins

To determine the roles of full-length New1p and Rnq1p in *[PSI⁺]* induction, we deleted the corresponding genes and tested the effect of these mutations on the ability of Sup-GFP to induce *[PSI⁺]*. We found that *[PSI⁺]* inducibility arose from novel, gain-of-function

properties of New-GFP and Rnq-GFP aggregates. Neither deletion of *NEW1* nor *RNQ1* rendered *[pin⁻]* yeast susceptible to *[PSI⁺]* induction by Sup-GFP alone (Table 1). Moreover, *[PSI⁺]* induction by dual over-expression of Sup-GFP together with New-GFP or Rnq-GFP still occurred in these deletion strains. Thus, New1p- and Rnq1p-mediated *[PSI⁺]* induction does not arise from loss of the activity of their corresponding full-length proteins. In contrast, deletion of *RNQ1* prevented the manifestation and propagation of the classical *[PIN⁺]* factor, a finding consistent with the identification of Rnq1p as the protein determinant of *[PIN⁺]* (Derkatch et. al, 2001). In Δ*new1* strains, there was a significant delay in the onset of the *[PSI⁺]* phenotype regardless of whether *[PSI⁺]* induction was promoted by New-GFP, Rnq-GFP or *[PIN⁺]* (Table 1 and data not shown). Once converted, *[PSI⁺]* Δ*new1* strains grew at a normal rate, suggesting that the chromosomally-encoded New1p assists in the induction but not propagation of *[PSI⁺]*.

Aggregation of New1p NYN-repeats is needed for *[PSI⁺]* induction

We next constructed a panel of truncated New1p-GFP fusions in order to investigate the relationship between the aggregation of New1p and its ability to promote *[PSI⁺]* induction. The prion domain of New1p contains a particularly Gln/Asn-rich region between

	Over-expressed Protein			Conversion to *[PSI⁺]*		
	Sup-GFP	New-GFP	Rnq-GFP	wild type	*Δnew1*†	*Δrnq1*
[pin⁻]	÷			-	-	-
		÷		-	-	-
			÷	-	-	-
	÷	÷		++	++	++
	÷		÷	+	+	+
[PIN⁺]	÷			++	++	-*

Table 1. Genetic analysis of New1- and Rnq1-mediated *[PSI⁺]* induction

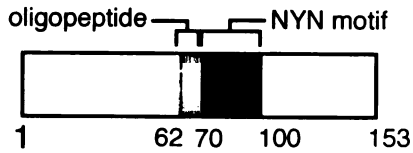
[psi⁻] strains of a *NEW1 RNQ1* (wild type), *Δnew1* or *Δrnq1* background over-expressing the indicated GFP fusion proteins were assayed for conversion to *[PSI⁺]* in the presence or absence of *[PIN⁺]* by growth on –ade. - indicates no conversion to *[PSI⁺]*, + indicates ~1% conversion and ++ represents ~10% conversion. All *Δnew1* experiments (†) showed delayed manifestation of *[PSI⁺]*; colonies appeared on –ade after ~14 days, instead of the 5 days seen with wild type strains.

* indicates permanent loss of the *[PIN⁺]* factor; unlike their wild type sister spores, *Δrnq1* haploids derived from a *[PIN⁺] Δrnq1/+* diploid failed to manifest *[PIN⁺]* as assayed by Sup-GFP aggregation and *[PSI⁺]* induction and did not transmit *[PIN⁺]* in subsequent back-crosses against *RNQ1 [pin⁻]* strains.

residues 50 and 100. Notably, residues 62-70 (QQGGYQSYN) resemble an oligopeptide repeat found in Sup35p (PQGGYQQYN) that influences the stability and severity of *[PSI+]* (Liu and Lindquist, 1999), while residues 71-100 of New1p contain the repeating tripeptide sequence NYN (Figure 2A).

We found that an intact NYN-rich region is essential for both New1p aggregation and its ability to promote *[PSI+]* induction (Figure 2B), whereas other portions of the New1p prion domain proved unnecessary for either effect. The C-terminal GFP domain was not required for *[PSI+]* inducibility, as over-expression an EE epitope-tagged version of the NYN region also promoted *[PSI+]* formation. Replacement of the first or third asparagine pairs of the NYN motif with large, positively charged residues (arginines) decreased both aggregation andconversion to *[PSI+]* (Figure 2C). Compared to an unmutated control, the distal arginine mutations diminished both the fraction of cells with aggregates and the rate of *[PSI+]* induction roughly six-fold; introduction of arginine residues near the center of the NYN motif completely abolished both the aggregation and *[PSI+]*-promoting properties of New1p. These data argue that susceptibility to *[PSI+]* induction arises from aggregation of New1p via its NYN repeat motif.

A



B

	foci	*[PSI+]* induction
GFP, 1–153	Yes	Yes
GFP, 1–50	No	No
GFP, 51–153	Yes	Yes
GFP, 101–153	No	No
GFP, 52–100	Yes	Yes
GFP, 52–70	No	No
GFP, 71–100	Yes	Yes
EE tag, 71–100	N/A	Yes

C

	Cells with NYN-GFP foci (%)	*[PSI+]* colonies (%)
(M)SNYNNYNNYNNYNNYNNYNNYNKYNGQGYQ...GFP	23.8	5.9
(M)SNY**RR**YNNYNNYNNYNNYNNYNKYNGQGYQ...GFP	3.9	1.0
(M)SNYNNYNNY**RR**YNNYNNYNNYNKYNGQGYQ...GFP	0	0

Figure 2. Mutational analysis reveals a relationship between New1p aggregation and [PSI$^+$] induction

(A) A schematic diagram of the New1p prion domain (residues 1-153). The oligopeptide region similar to Sup35p (QQGGYQSYN, residues 62-70, gray) and the NYN repeat motif (residues 70-100, black) are indicated.

(B) NYN motif of New1p confers both aggregation and *[PSI$^+$]* inducibility. Fusions between the indicated regions of New1p and GFP were assayed for focus formation (visualized by fluorescent microscopy) when over-expressed alone, as well as the ability to facilitate *[PSI$^+$]* induction when over-expressed along with Sup-GFP. The New$_{70\text{-}100}$-EE construct was ~10 times less effective than the New-GFP construct at inducing *[PSI$^+$]* in conjunction with Sup-GFP, possibly due to poor expression.

(C) Disruption of the NYN motif affects aggregation and *[PSI$^+$]* inducibility. Pairs of arginines (denoted RR) were introduced in place of asparagines pairs at the indicated positions in the NYN tract of New1$_{70\text{-}100}$-GFP. The percentage of cells containing visible GFP inclusions and cells converted to *[PSI$^+$]* are shown.

The *[NU⁺]* prion confers susceptibility to *[PSI⁺]* induction

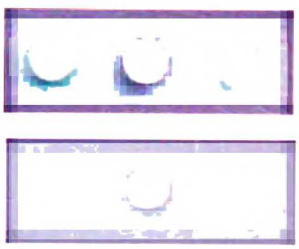
The preceding experiments linked *[PSI⁺]* inducibility to the presence of New-GFP aggregates produced by over-expression. We asked whether low levels of a constitutively aggregated form of the New1p prion domain could also confer *[PSI⁺]* inducibility. To generate pure populations of cells containing such aggregates without a need for continuous New-GFP over-expression, we replaced the chromosomal copy of *SUP35* with an episomal gene encoding a fusion of the New1p prion domain and the essential translation termination domain of Sup35p. In the resulting strains, this protein (termed New-EF) could be inter-converted from a soluble *[nu⁻]* form to the self-propagating aggregated state, *[NU⁺]* (Santoso et al., 2000). In *[NU⁺]* strains, the majority of chromosomally encoded New1p protein is also aggregated (data not shown). As with *[PSI⁺]*, the *[NU⁺]* state could be monitored phenotypically by nonsense suppression of *ade1-14*, resulting in adenine prototrophy.

We first established the prion nature of New-EF aggregates responsible for *[NU⁺]*. As with other yeast prions, transient treatment of *[NU⁺]* cells with guanidine caused efficient reversion to the *[nu⁻]*

state (Figure 3A). Similarly, we were unable to obtain *[NU$^+$]* isolates in strains lacking Hsp104p, which is thought to be the target of guanidine (Jung and Masison, 2001) (data not shown). New-EF protein is required for the propagation of *[NU$^+$]*, as transient loss of the New-EF-expressing plasmid caused reversion to *[nu$^-$]* (Figure 3B). Finally, *[NU$^+$]* can be transmitted in an "infectious" manner through cytoplasmic transfer (cytoduction) from *[NU$^+$]* cells to karyogamy-defective *[nu$^-$]* cells (Figure 3C). Whereas *[NU$^+$]* donors efficiently transferred adenine prototrophy to *[nu$^-$]* recipients, no ADE+ colonies were obtained when *[nu$^-$]* donors were used. Combined with previously published data (Santoso et al., 2000), these results confirmed that *[NU$^+$]* results from an infectious, reversible, conformational isoform (i.e. prion) of the New-EF protein.

We next created a panel of strains that harbored one, both or neither of the *[PIN$^+$]* or *[NU$^+$]* prions and used fluorescence microscopy to monitor the formation of Sup-GFP aggregates, which appeared as both foci and "ribbons" (Figure 4A). Although it is unknown whether these structures are active *[PSI$^+$]* seeds, their formation depends on the induction or presence of *[PSI$^+$]* and thus serves as an indicator of *[PSI$^+$]* susceptibility (Patino et al., 1996; Zhou et al., 2001).

A

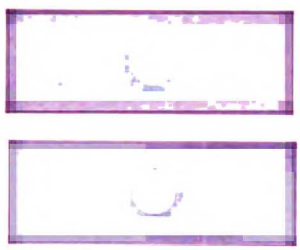


low ade

no ade

[nu⁻] *[NU+]* *[NU+]* after GuHCl

B



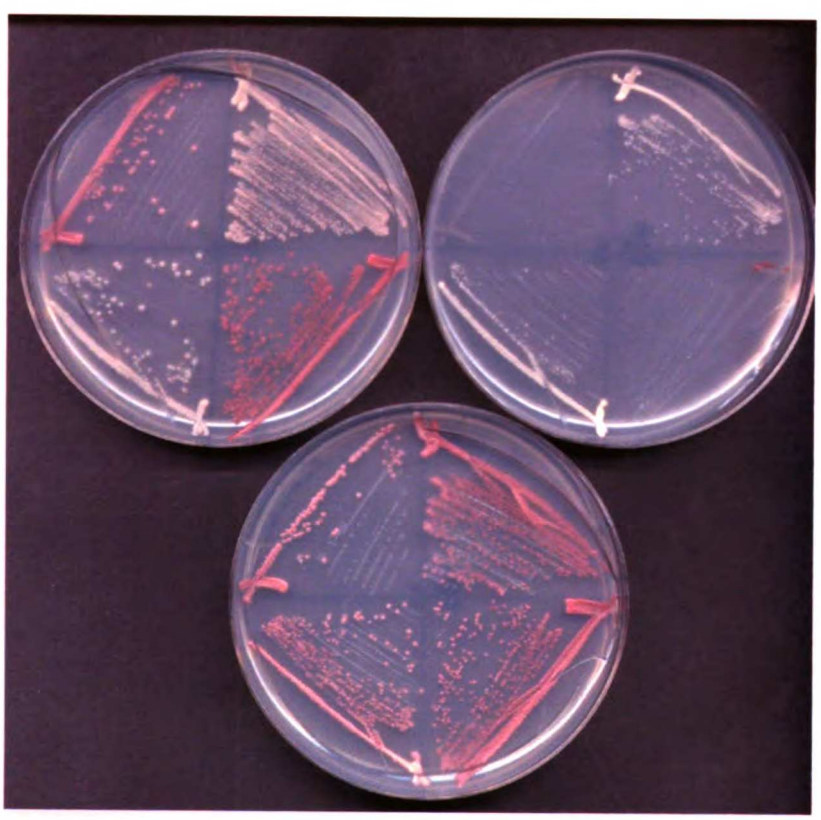
low ade

no ade

[nu⁻] *[NU+]* *[NU+]* after shuffle

C

low ade



no ade

low ade + GuHCl

Legend

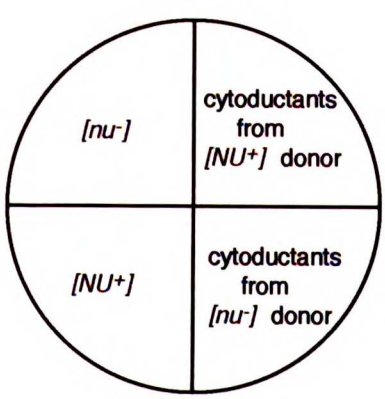


Figure 3. *[NU$^+$]* is a prion form of New-EF

(A) *[NU$^+$]* is eliminated by guanidine treatment. Shown are spotted cultures of *[nu$^-$]* and *[NU$^+$]* strains and the *[NU$^+$]* strain after transient growth (~20 generations) in the presence of guanidine. The strains shown here and in subsequent panels are *[PIN$^+$]*, but similar results were obtained with *[pin$^-$]* derivatives.

(B) Propagation of *[NU$^+$]* requires the continuous expression of the New-EF protein. *[NU$^+$]* strains bearing an episomal copy of New-EF were transformed with a plasmid encoding Sup35p and were allowed to lose the New-EF plasmid. These isolates were then re-tranformed with the New-EF plasmid and were allowed to lose the *SUP35* plasmid. When tested for *ade1-14* nonsense suppression after this plasmid "shuffle," the resulting strains had lost the *[NU$^+$]* trait.

(C) *[NU$^+$]* is efficiently transmitted by cytoplasmic transfer (cytoduction). Cytoductants from *[nu$^-$]* or *[NU$^+$]* donor strains to a karyogamy-deficient *[nu$^-$]* recipient are shown growing on media with low or no adenine (top). Cytoductable adenine prototrophy was abolished by growth on medium with guanidine (bottom), confirming its prion basis. Shown are streaks from non-clonal cytoductant patches; when individual cytoductant colonies were scored for *[NU$^+$]*, 17 of 17 colonies obtained using a *[NU$^+$]* donor had become *[NU$^+$]*,

while 17 of 17 colonies from *[nu⁻]* donor remained *[nu⁻]*. For comparison, the *[NU⁺]* and *[nu⁻]* donor stains are also shown.

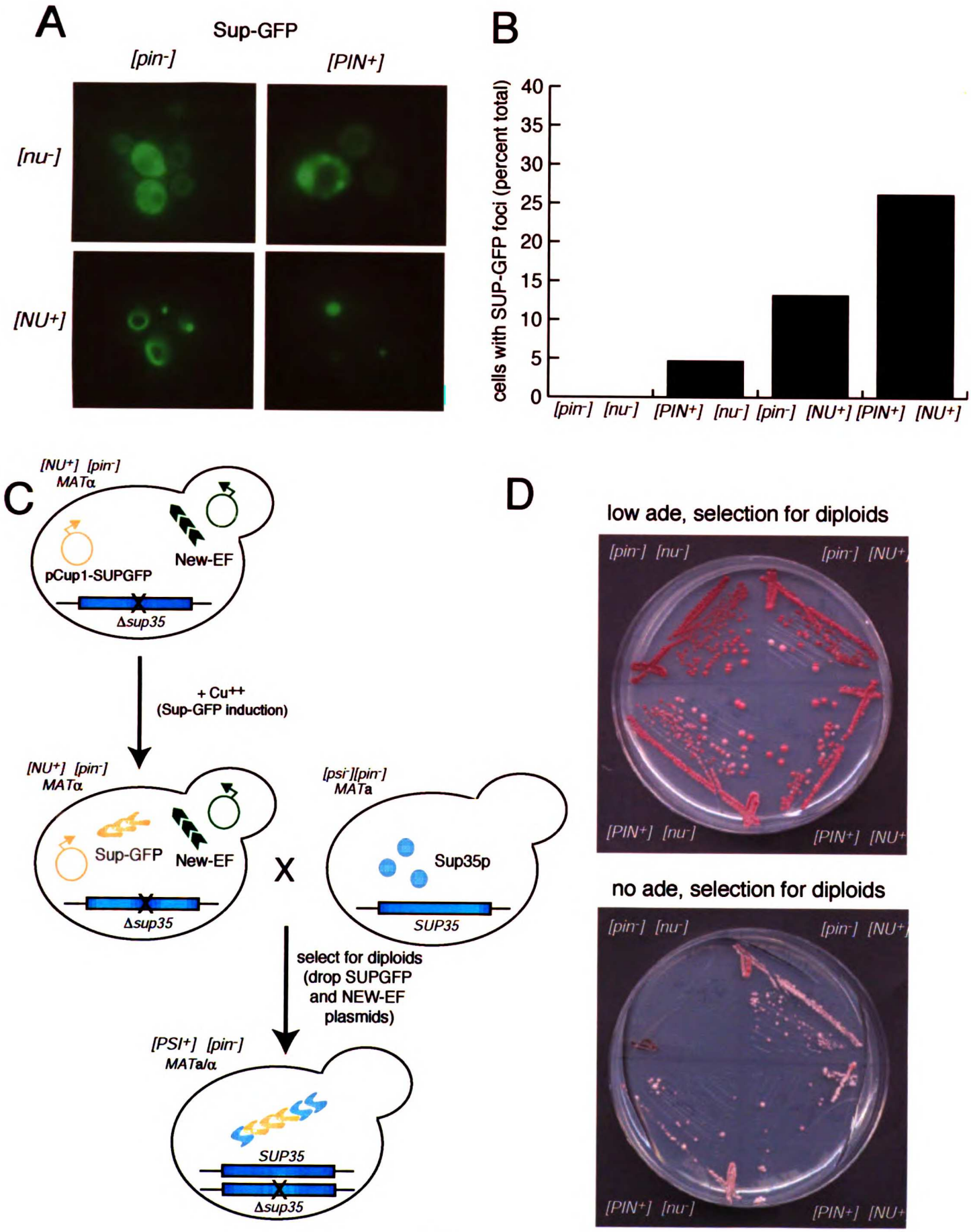

A
Sup-GFP
[pin-]
[PIN+]
[nu-]
[NU+]
B
cells with SUP-GFP foci (percent total)
40
35
30
25
20
15
10
5
0
[pin-] [nu-] [PIN+] [nu-] [pin-] [NU+] [PIN+] [NU+]
C
[NU+] [pin-]
MATα
pCup1-SUPGFP
New-EF
Δsup35
+ Cu++
(Sup-GFP induction)
[NU+] [pin-]
MATα
Sup-GFP
New-EF
Δsup35
X
[psi-][pin-]
MATa
Sup35p
SUP35
select for diploids
(drop SUPGFP
and NEW-EF
plasmids)
[PSI+] [pin-]
MATa/α
SUP35
Δsup35
D
low ade, selection for diploids
[pin-] [nu-]
[pin-] [NU+]
[PIN+] [nu-]
[PIN+] [NU+
no ade, selection for diploids
[pin-] [nu-]
[pin-] [NU+]
[PIN+] [nu-]
[PIN+] [NU+

Figure 4. *[NU$^+$]* confers susceptibility to *[PSI$^+$]* induction

(A) Sup-GFP forms aggregates in *[PIN$^+$]* or *[NU$^+$]* cells. Sup-GFP was expressed from a copper-inducible high-copy plasmid in strains harboring neither, one or both of the *[PIN$^+$]* and *[NU$^+$]* prions, as indicated. After 48 hours of expression, foci and ribbons of fluorescence were visible in cells harboring at least one prion. In this and subsequent fluorescence localization experiments (all of which were performed under similar conditions), ~50% of cells had little or no detectable fluorescence; non-fluorescent cells were nonetheless included in all quantitative analyses.

(B) Frequency of Sup-GFP focus formation in cells treated as described in (A).

(C) Scheme for testing the ability of pre-formed Sup-GFP aggregates to seed prion conversion of full-length Sup35p. Chromosomal *SUP35* (blue) was disrupted, allowing *[NU$^+$]* to be monitored by inactivation of the New-EF protein (green). Aggregates of Sup-GFP (orange) were accumulated by copper-induced over-expression from the pCup1-SUPGFP plasmid (top left). The cells were then mated with a *[psi$^-$] [pin$^-$]* strain expressing full-length Sup35p (middle). The *[PSI$^+$]* state of the resulting diploid was assayed by growth on –ade. The appearance of *[PSI$^+$]* indicated that Sup-GFP aggregates had seeded the prion conversion of full-length

Sup35p (bottom). Similar experiments were performed starting with cells lacking *[PSI⁺]*-promoting prions, with *[PIN⁺]* alone or with both *[NU⁺]* and *[PIN⁺]*. In all cases, diploids were plated on medium that allowed the loss of the Sup-GFP- and New-EF-encoding plasmids.

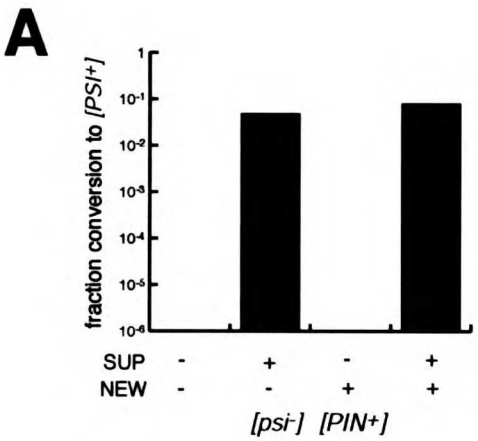
(D) Demonstration that Sup-GFP aggregates formed as a result of the presence of *[NU⁺]* and *[PIN⁺]* can seed *[PSI⁺]* induction. Cells with neither, one or both of the *[PIN⁺]* and *[NU⁺]* prions were treated as described in (C). The top panel (low ade) shows the growth of all diploids, while the bottom panel (no ade) shows the growth of *[PSI⁺]* diploids only. Note lack of growth in top left quadrant of bottom panel.

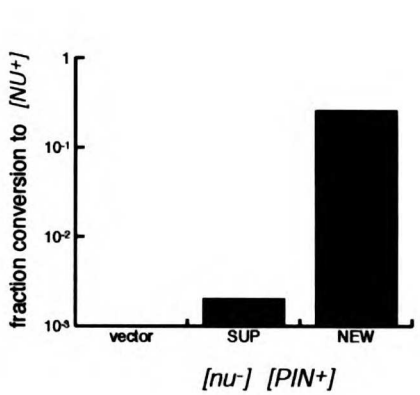
We found that *[NU$^+$]* conferred susceptibility to *[PSI$^+$]* induction in a manner that was comparable to but independent of *[PIN$^+$]*. *[NU$^+$]* alone promoted the appearance of Sup-GFP foci at an efficiency somewhat higher than *[PIN$^+$]* alone, and in combination, *[NU$^+$]* and *[PIN$^+$]* resulted in a further increase in the formation of foci (Figure 4B). By contrast, no Sup-GFP aggregates were seen in cells lacking both prion elements, including *[NU$^+$] [pin$^-$]* strains that spontaneously reverted to *[nu$^-$] [pin$^-$]* (Figure 4B and data not shown). We directly demonstrated the presence of prion seeds in cells with such Sup-GFP aggregates by using them to seed the conversion of full length Sup35p to a *[PSI$^+$]* state. We first accumulated Sup-GFP aggregates in D*sup35* strains, then exposed these aggregates to full length Sup35p through mating with a *[psi$^-$] [pin$^-$]* strain with an intact *SUP35* gene (depicted in Figure 4C); the resulting diploids were assayed for conversion to *[PSI$^+$]*. Only donor strains with visible Sup-GFP aggregates prior to mating (i.e, strains with either the *[NU$^+$]* or *[PIN$^+$]* prions) yielded *[PSI$^+$]* diploid colonies (Figure 4D). Taken together, these findings establish that prion-facilitated *[PSI$^+$]* inducibility can arise from the presence of multiple independent prions.

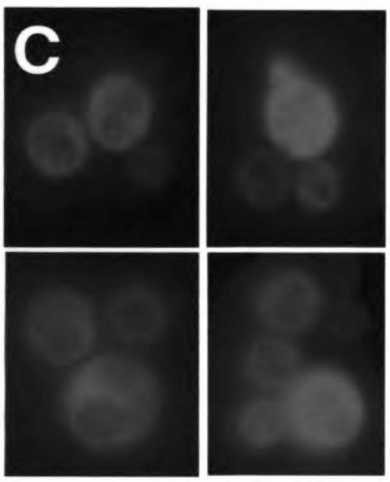
New1p aggregates do not seed Sup35p polymerization

New1p or Rnq1p prions could promote *[PSI⁺]* induction by providing a nucleating seed into which Sup35p could become incorporated. However, several experiments designed to detect such cross-seeding yielded negative results. First, New-GFP over-expression did not by itself stimulate *[PSI⁺]* induction, even in an already susceptible *[PIN⁺]* strain (Figure 1A and Figure 5A). Conversely, Sup35p aggregates produced by over-expressing Sup-GFP in a *[PIN⁺]* strain did not increase the rate of appearance of *[NU⁺]* (Figure 5B). Additionally, over-expression of Sup35p did not induce *[PIN⁺]* (Derkatch et al., 1997). Finally, the presence of purified recombinant New1p prion domain did not affect the kinetics of Sup35p prion domain polymerization in vitro (data not shown).

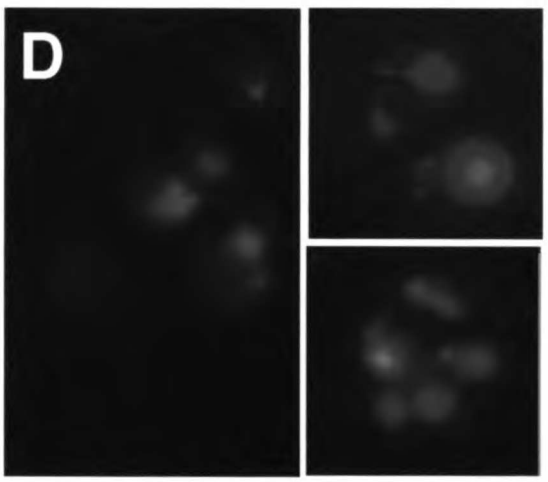
To test directly for cross-interaction between Sup35p and New1p prion domains in vivo, we examined the localization of these proteins in the same cells using two-color fluorescence microscopy. A fusion of the Sup35p prion domain with yellow fluorescent protein (Sup-YFP) displayed diffuse cytoplasmic localization when over-expressed alone in a *[pin⁻]* strain (Figure 5C); in contrast, a New1p prion domain—cyan fluorescent protein fusion (New-CFP) formed



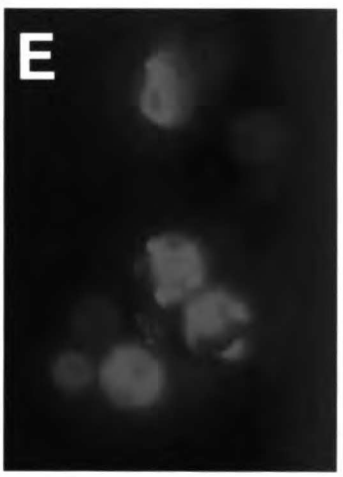




[psi-] [pin-]
Sup-YFP
yellow



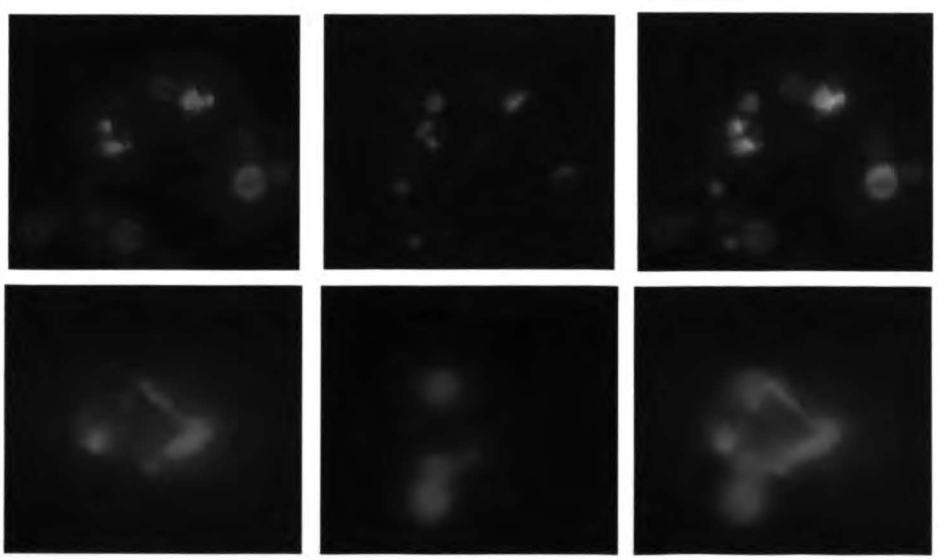
[psi-] [pin-]
New-CFP
cyan



[psi-] [pin-]
Sup-YFP
New-CFP
yellow

F

[psi-] [pin-]
Sup-YFP
New-CFP



yellow cyan merge

Figure 5. Lack of cross-interaction between New-GFP and Sup-GFP

(A) New-GFP aggregates do not induce *[PSI$^+$]*. The indicated GFP fusion proteins were over-expressed in a *[psi$^-$] [PIN$^+$]* strain and the frequency of conversion to *[PSI$^+$]* was determined as in Figure 1A.

(B) Sup-GFP aggregates do not induce *[NU$^+$]*. The indicated GFP fusion proteins were over-expressed in a *[nu$^-$] [PIN$^+$]* strain. Conversion to *[NU$^+$]* was monitored by nonsense suppression resulting from aggregation of New-EF protein. Note that the spontaneous frequency of appearance of *[NU$^+$]* is ~10^{-3}, significantly higher than that of *[PSI$^+$]*.

(C) Sup-YFP over-expressed alone in a *[psi$^-$] [pin$^-$]* strain displays diffuse localization. In these and subsequent micrographs, the prion status, expressed proteins and the fluorescent light channel are indicated.

(D) New-CFP forms aggregates when over-expressed in *[psi$^-$] [pin$^-$]* strains. The majority of cells with visible fluorescence had inclusions, as shown here.

(E) Sup-YFP forms filamentous aggregates in a subset of cells when over-expressed together with New-CFP in a *[psi$^-$] [pin$^-$]* strain.

(F) New-CFP and Sup-YFP aggregates do not co-localize. A field view with yellow, cyan and merged channels is shown at top, while the bottom row shows a magnified view from a separate experiment.

In all cells with Sup-YFP inclusions, New-CFP inclusions were also observed. Lack of co-localization was confirmed by 3D reconstruction of wide-field microscopic images (O. Weiner and L.Z.O., data not shown).

aggregates when over-expressed alone in the same strain (Figure 5D). When these proteins were over-expressed together, 2-5% of the cells displayed aggregates of Sup-YFP (Figure 5E) as well as of New-CFP, but these two types of inclusions did not co-localize (Figure 5F). Thus, the presence of New-CFP permitted the ordinarily soluble Sup-YFP to aggregate, but not into the same inclusions as New-CFP.

The role of chaperones in [PSI$^+$] induction

Another possible basis for New1p-mediated *[PSI$^+$]* induction might involve changes in the activities of chaperones that modulate *[PSI$^+$]*. The presence of aggregated New1p could in principle elevate Hsp104p activity, which has been suggested to stimulate the folding of the *[PSI$^+$]* form of Sup35p. However, we found that New-CFP promoted Sup-YFP aggregation even in the absence of Hsp104p. In a strain lacking Hsp104p, Sup-YFP displayed diffuse localization when over-expressed alone (Figure 6A), while New-CFP formed aggregates (Figure 6B) similar to those seen in wild type controls (Figure 5D). As in wild type cells, the presence of New-CFP permitted Sup-YFP to aggregate (Figure 6C). Over-expression of chaperones also did not prevent New-GFP from stimulating *[PSI$^+$]*

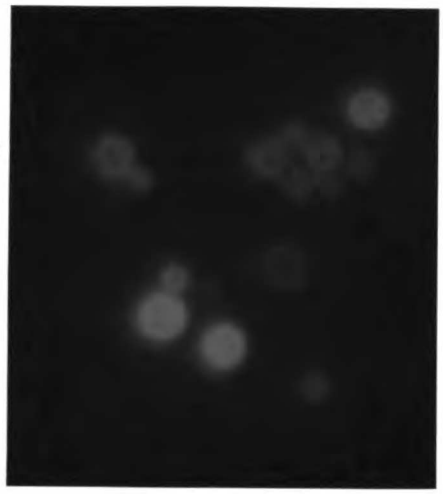
induction. We expressed New-GFP and Sup-GFP in a strain bearing a high copy *HSP104* plasmid previously shown to cure *[PSI⁺]*, but this did not have a substantial effect on the ability of New-GFP to promote *[PSI⁺]* induction (Figure 6D). We performed similar experiments with high copy plasmids encoding Ssa1p and Ssb1p, two Hsp70 proteins known to affect *[PSI⁺]* formation [Newnam, 1999 #46;Chernoff, 1999 #44], and obtained similar results (data not shown). These observations indicate that the effect of New1p aggregates is not mitigated by altering levels of chaperones known to modulate *[PSI⁺]*. Furthermore, they suggest that Hsp104p, which is absolutely required for the propagation of *[PSI⁺]*, is not needed for the *de novo* formation of Sup35p aggregates.

[PSI⁺] inducibility factors promote polyGln aggregation in yeast

Finally, we asked whether the presence of *[PSI⁺]*-promoting prions affected the aggregation of Gln/Asn-rich proteins other than Sup35p. We constructed GFP fusions of normal and glutamine-expanded variants of a fragment of the spinocerebellar ataxia type 3/Machado-Joseph Disease (MJD) protein (Ikeda et al., 1996) and over-expressed them in *[pin⁻]* and *[PIN⁺]* yeast. As expected, fusions with 22 glutamines (Q22-GFP) did not aggregate in either strain. In

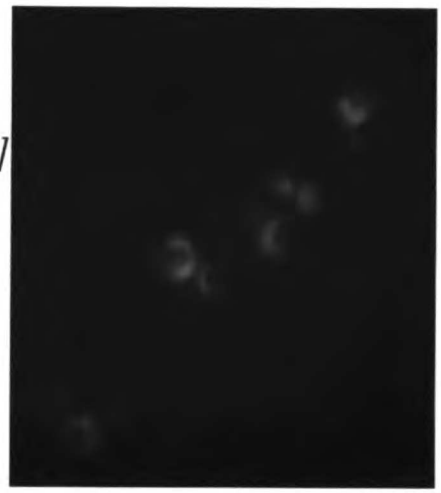
A

[psi-] [pin-]
Δhsp104
Sup-YFP
yellow



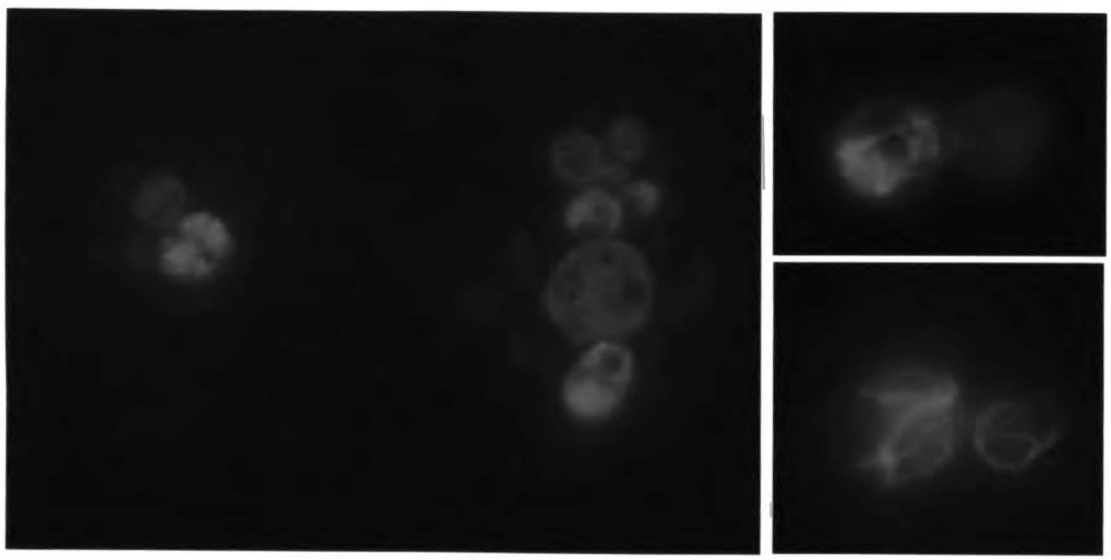
B

[psi-] [pin-]
Δhsp104
New-CFP
cyan

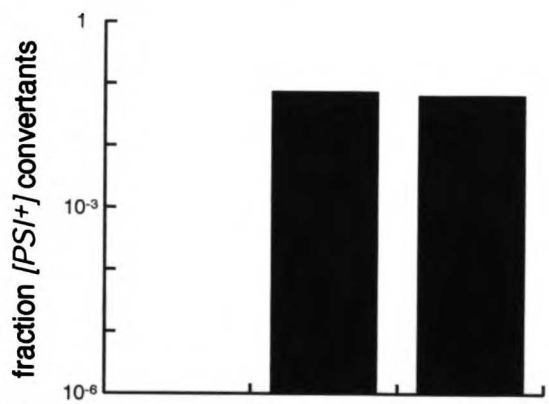


C

[psi-] [pin-]
Δhsp104
Sup-YFP
New-CFP
yellow



D



OE			
Sup-GFP	+	+	+
New-GFP	-	+	+
Hsp104p	-	-	+

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Figure 6. Altered Hsp104p levels do not affect New1-mediated Sup35p aggregation or *[PSI⁺]* induction

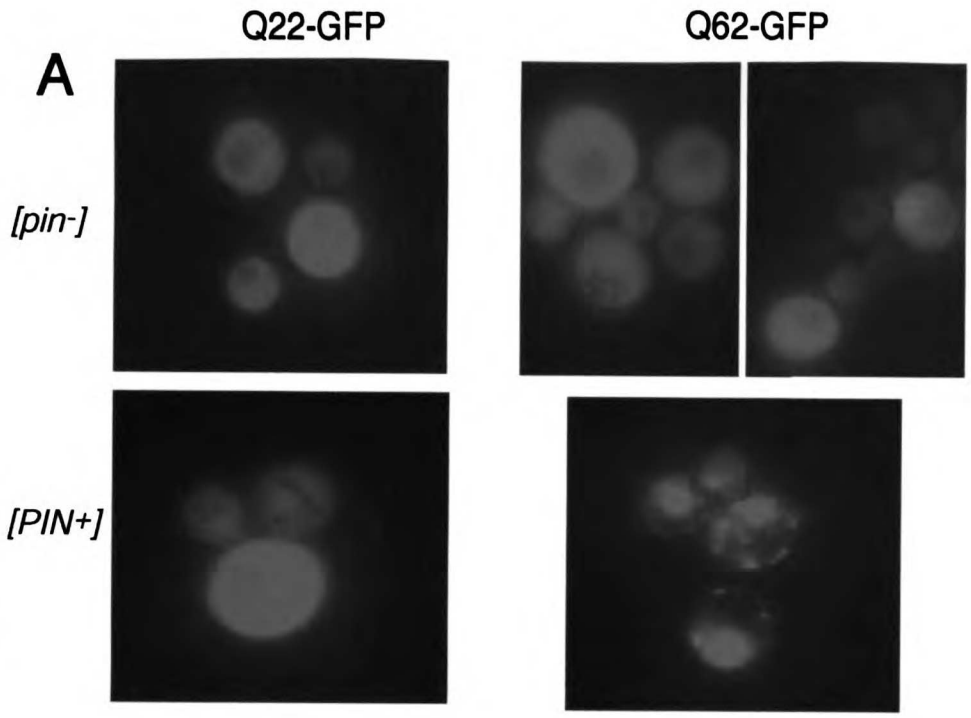
(A) Examples of the diffuse localization of Sup-YFP observed in a D*hsp104* strain in the absence of New-CFP over-expression.

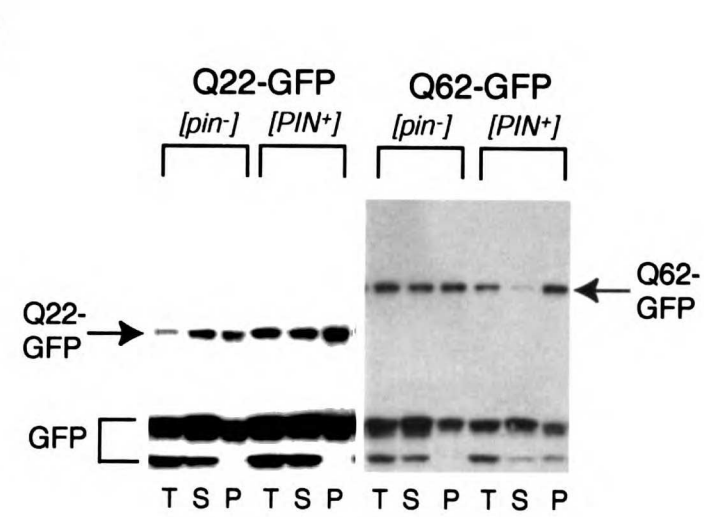
(B) Examples of New-CFP aggregates in a Δ*hsp104* strain.

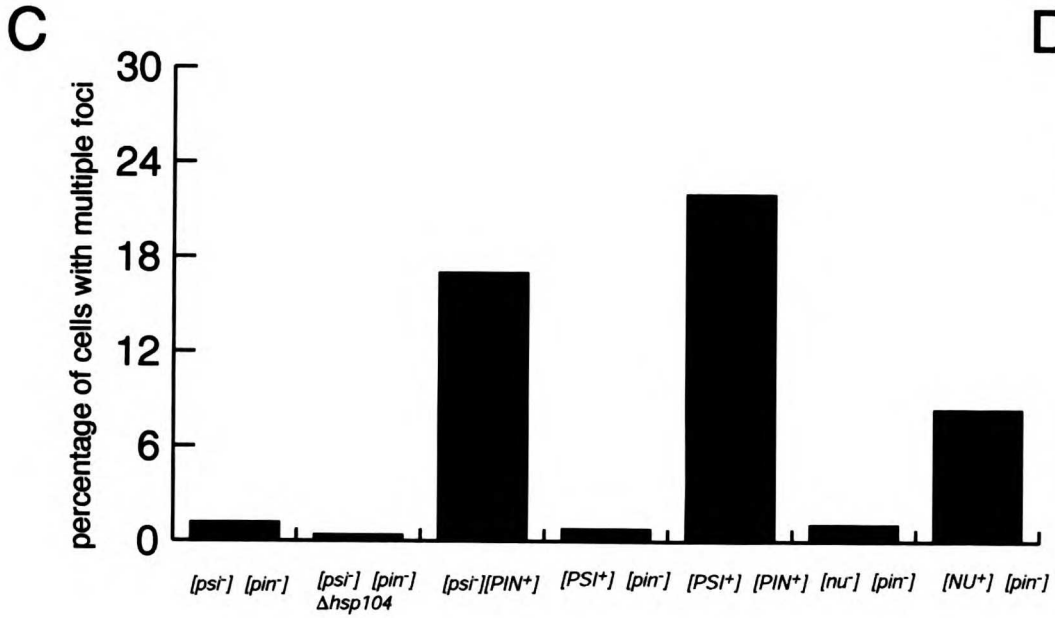
(C) New-CFP-mediated aggregation of Sup-YFP does not require Hsp104p. A *[psi⁻] [pin⁻] Δhsp104* strain over-expressing both Sup-YFP and New-CFP displayed Sup-YFP inclusions at a frequency (~2%) similar to that in *[psi⁻] [pin⁻]* cells with intact *HSP104* (see Figure 5E).

(D) Hsp104p over-expression does not affect New1p-mediated *[PSI⁺]* induction. *[psi⁻] [pin⁻]* strains with or without a high copy *HSP104* plasmid were plated onto -ade after over-expression of the indicated GFP fusion proteins. When these strains were plated onto -ade while still selecting for the *HSP104* plasmid, no ADE+ colonies were observed, confirming that over-expressed *HSP104* could prevent *[PSI⁺]* propagation (data not shown).

contrast, a fusion containing a pathogenic number of glutamines (Q62-GFP) formed visible foci, but the number and nature of these aggregates was greatly altered by the presence of *[PIN$^+$]* (Figure 7A-C). In strains in which *[PIN$^+$]* had been eliminated by guanidine treatment or deletion of *HSP104*, Q62-GFP fluorescence was predominantly diffuse, with some cells (~6%) exhibiting a single large inclusion (Figure 7A top right); cells containing multiple foci were very rare (~1%). However, in a *[PIN$^+$]* strain, there were few cells with bright diffuse fluorescence; cells with visible aggregates were more abundant (~28%) and most of these cells (~18% of total) exhibited multiple minute foci (Figure 7A bottom right). We obtained similar results with a MJD protein-GFP fusion containing 82 glutamines (data not shown). Biochemical analysis confirmed that the *[PIN$^+$]* element promoted the aggregation of Q62-GFP, as this protein was depleted by ultra-centrifugation from the supernatant of *[PIN$^+$]* but not *[pin$^-$]* extracts (Figure 7B). Like *[PIN$^+$]*, *[NU$^+$]* stimulated the appearance of multiple fluorescent foci of Q62-GFP (Figure 7C and 7D). However, *[PSI$^+$]* did not promote the formation of Q62-GFP foci (Figure 7C). Together, these observations reveal a role for *[PSI$^+$]*-promoting prions in the general regulation of protein aggregation and suggest a common mechanism for the initiation of Sup35p and polyGln disease protein aggregates.







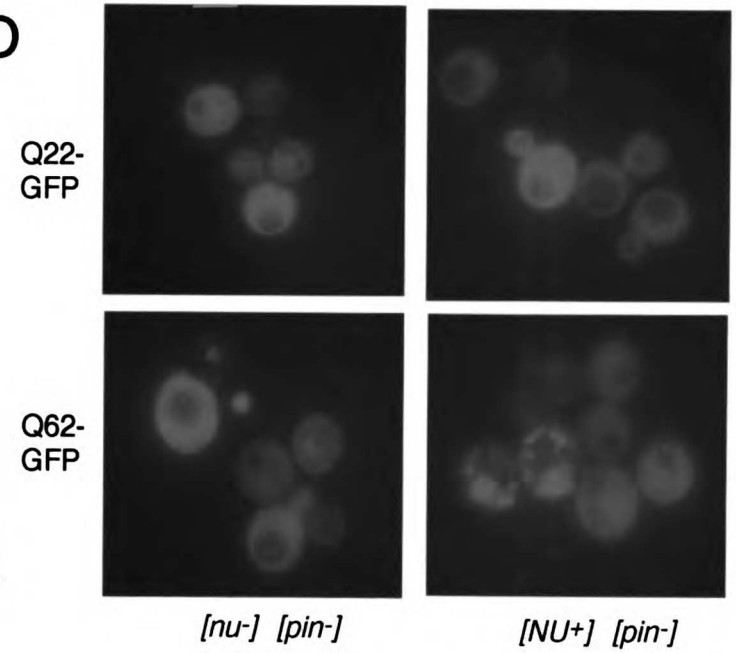


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Figure 7. *[PIN$^+$]* and *[NU$^+$]* promote aggregation of the polyGln disease protein MJD/ataxin-3

Q22 and Q62 refer to MJD protein-GFP fusions containing 22 and 62 Gln residues, respectively.

(A) Representative fluorescence micrographs illustrating the effect of glutamine repeat length and the *[PIN$^+$]* prion on MJD-GFP aggregation. Q22 and Q62 were over-expressed in *[psi$^-$] [pin$^-$]* and *[psi$^-$] [PIN$^+$]* strains. Note the single large inclusion (top right) seen in a subset of *[pin$^-$]* Q62 cells; in contrast, *[PIN$^+$]* Q62 cells displayed more abundant multiple aggregates (bottom right).

(B) Centrifugation analysis of MJD-GFP. Lysates prepared from the indicated strains over-expressing either Q22 or Q62 were subjected to ultra-centrifugation and the total (T), supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE followed by immunoblotting with a-GFP. Q22 is distributed equally between the supernatant and the pellet in both strains, while Q62 sediments almost entirely to the pellet of the *[PIN$^+$]* extract but remains soluble in the *[pin$^-$]* extract. The smaller bands (GFP) likely result from proteolytic removal of the MJD region; as expected, these fragments are not depleted from the supernatant.

(C) Effect of various yeast prions on Q62 aggregation. For each of the indicated strains, the fraction of cells with multiple fluorescent

foci was determined as in Figure 3A. The lower stimulation of aggregation of Q62 by *[NU$^+$]* compared to *[PIN$^+$]* may result from the rapid reversion of these strains to *[nu$^-$]* under these conditions (data not shown). Note that *[PSI$^+$]* does not stimulate Q62 aggregation.

(D) Representative *[nu$^-$] [pin$^-$]* and *[NU$^+$] [pin$^-$]* cells over-expressing Q22 and Q62.

Discussion

Susceptibility to induction of the yeast *[PSI⁺]* prion has previously been shown to require a non-Mendelian element, *[PIN⁺]*, which itself propagates in a prion-like manner (Derkatch et al., 2000; Derkatch et al., 1997). We have investigated the role of two Gln/Asn-rich prion domains, those of New1p and Rnq1p, in the process of *[PSI⁺]* induction. Our studies reveal that the prion forms of these proteins control not only susceptibility to *[PSI⁺]* formation but also enhance the aggregation of a fragment of a human disease protein (MJD) containing a pathogenic number of glutamine repeats. Additionally, we have described a new mode of yeast prion action that, as with the mammalian prions, arises from gain-of-function properties of protein aggregates rather than from the inactivation of the normal protein (Prusiner, 1998; Wickner et al., 1999).

We have established that while susceptibility to *[PSI⁺]* induction can be conferred by at least two yeast prion domains, it is not an obligatory consequence of the accumulation of intracellular Gln/Asn-rich aggregates. *[PSI⁺]* inducibility is widespread among laboratory yeast strains (L.Z.O. and J.S.W., unpublished data), as is the presence of Rnq1p aggregates (Sondheimer and Lindquist, 2000); indeed, an accompanying paper demonstrates that the prion

form of Rnq1p is responsible for the classical *[PIN⁺]* state (Derkatch et al., 2001). Given the large number of other potential prions in the yeast genome (Michelitsch and Weissman, 2000), it seems likely that aggregates of additional, as-yet undiscovered prions will also confer susceptibility to *[PSI⁺]* induction.

Several observations suggest a common mechanism of action by the prion forms of New-EF (*[NU⁺]*) and Rnq1p (*[PIN⁺]*). Both prions render the cell susceptible to *[PSI⁺]* induction and enhance polyGln aggregation. Furthermore, *[PSI⁺]* susceptibility arising from either prion is partially compromised by deletion of the chromosomal *NEW1* gene. Finally, Sup-GFP aggregates generated by virtue of *[NU⁺]* and *[PIN⁺]* are microscopically indistinguishable and lead to a similar range of *[PSI⁺]* prion "strains" (Derkatch et al., 1996).

A saturable anti-aggregation system?

How do aggregates of New1p or Rnq1p make cells vulnerable to *[PSI⁺]* induction? Our findings exclude several models. First, *[PSI⁺]* induction does not occur as a result of the *de novo* appearance of the *[PIN⁺]* factor. Second, *[PSI⁺]* induction does not arise through loss of functional New1p or Rnq1p; deletion of these genes did not confer *[PSI⁺]* inducibility. Third, New1p-mediated *[PSI⁺]* susceptibility is not solely caused by changes in Hsp104p

activity, as neither loss nor over-expression of Hsp104p modulated this phenomenon.

[PSI$^+$] susceptibility could arise from cross-seeding between aggregates of Sup35p and those of New1p and Rnq1p prion domains. Several observations argue against this model. Sup35p prion propagation is highly self-specific; even single amino acid changes can prevent cross-seeding between wild type and mutant Sup35p proteins (DePace et al., 1998). While a small portion of New1p does resemble an oligopeptide repeat sequence found in Sup35p, this region proved dispensable for *[PSI$^+$]* induction. Furthermore, New-GFP over-expression alone failed to induce *[PSI$^+$]* and Sup35p over-expression did not stimulate *[NU$^+$]* induction. Finally, Sup-YFP and New-CFP aggregates in the same cell did not co-localize. Despite these findings, it remains possible that some cross-seeding could occur at a low level and we are unable to conclusively rule out such a basis for the observed phenomena.

As an alternative model, we suggest that there may exist saturable cellular factors that antagonize *de novo* formation of prion-like aggregates. In a prion-free (*[psi$^-$] [pin$^-$]*) strain, over-expressed Sup35p could be recognized by this anti-aggregation factor and prevented from aggregating. Aggregates of Rnq1p or New1p, whether arising from over-expression or the presence of

heritable prions, could specifically inhibit this anti-aggregation factor, thereby allowing Sup35p to form the *[PSI⁺]* prion.

Chaperones and proteases can inhibit protein aggregation (Kopito, 2000), and are thus attractive candidates for saturation by *[PSI⁺]*-promoting factors. Although over-expression of several chaperones known to affect *[PSI⁺]* propagation did not appear to influence *[PSI⁺]* induction, multiple chaperones may be simultaneously inhibited by *[PSI⁺]*-promoting prions. For example, it has been shown that Rnq1p aggregates interact with an Hsp40/Hsp70 protein pair (Sis1p and Ssa1p) (Sondheimer et al., 2001). Alternatively, degradation of aggregation-prone proteins by cellular proteases could be inhibited by prion-promoting aggregates; polyGln-rich aggregates have in fact been recently found to saturate the proteasome (Bence et al., 2001). Preliminary data suggest that Sup-GFP is subject to amino-terminal proteolytic processing, but over-expression of New-GFP or the presence of *[PIN⁺]* antagonizes this effect (L.Z.O. and J.S.W., unpublished data). This inhibition of proteolysis occurs even with a mutant Sup-GFP (DePace et al., 1998) that does not aggregate, arguing that this protection from proteolysis is not merely a consequence of Sup35p aggregation.

Our findings also shed light on the role of Hsp104p in the induction and maintenance of *[PSI⁺]*. To explain the dependence of

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[PSI⁺] on Hsp104p, two models (which are not mutually exclusive) have been proposed. Hsp104p could assist the folding of Sup35p into an aggregation-competent state required for *[PSI⁺]* formation (Patino et al., 1996). Alternatively, the dissaggregation activity of Hsp104p may be needed to split up Sup35p aggregates to allow efficient distribution of the prion to daughter cells in cell division (Kushnirov and Ter-Avanesyan, 1998). Because deletion of *HSP104* eliminates all known yeast prions including *[PIN⁺]*, it has hitherto been impossible to determine whether Hsp104p is needed for the formation of Sup35p prion aggregates. However, over-expression of New1p circumvents the requirement for *[PIN⁺]* in *[PSI⁺]* induction, allowing us to test the effect of *HSP104* deletion on *de novo* Sup35p aggregation. We found that Sup-YFP in cells with aggregated New-CFP forms visible inclusions of similar appearance and frequency in both *Δhsp104* and wild type strains, demonstrating that Hsp104p is not needed for Sup35p aggregation *per se.* However, because Hsp104p is required for propagation of *[PSI⁺]*, we were not able to directly test whether these aggregates were prions.

Implications for human polyGln protein aggregation

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A set of neurodegenerative diseases, including spinocerebellar ataxia type 3/Machado-Joseph Disease (MJD) and Huntington's Disease, result from the expansion of glutamine repeats that cause the affected proteins to form intracellular inclusions. We have found that, in yeast, the *[PIN$^+$]* and *[NU$^+$]* prions strongly modulate the aggregation of a fragment of the MJD protein bearing pathogenic glutamine tracts. Recently it was demonstrated that deletion of *HSP104* abolishes the aggregation of glutamine-expanded Huntington's Disease protein in a yeast model (Krobitsch and Lindquist, 2000). Our results suggest that this requirement for *HSP104* may in part reflect the role of this chaperone in the maintenance of *[PIN$^+$]* or possibly other unidentified prions that promote polyGln aggregation.

The stimulation of MJD protein aggregation by *[PIN$^+$]* and *[NU$^+$]* establishes that the aggregation-promoting effect of these prions is not idiosyncratic to Sup35p but rather that they can also enhance the formation of polyGln aggregates, which are not known to be prion-like in character. A surprisingly large number of Gln/Asn-rich domains (~1% of total genomic ORFS) are encoded by eukaryotic genomes (Michelitsch and Weissman, 2000). Given the tendency of proteins of this type to aggregate, it is likely that cells seek to prevent such potentially deleterious misfolding. Differences

in the level of anti-aggregation activity from one cell type to another could contribute to the tissue specificity of polyGln diseases. Pathogenic aggregates may develop spontaneously in cells with a lower capacity to inhibit the initiation of polyGln aggregates. Once established, Gln/Asn-rich aggregates are likely to be difficult to eliminate and could tax the cell's ability to prevent other proteins from aggregating. Further studies of the initiation of aggregation in yeast and higher eukaryotes may thus prove fruitful in the identification of targets for prophylaxis against aggregation-based neurodegenerative diseases.

Experimental Procedures

Yeast Strains and Methods

Strains; *[PSI$^+$]* and *ade1-14* were introduced into W303 yeast by mating against the strain 74D-694**a** (Chernoff et al., 1995), followed by four back-crosses against the W303 parent, ultimately producing YJW 508 (*[PSI$^+$] [PIN$^+$] MATα*, *ade1-14*, *his3-11,15*, *leu2-3*, *trp1-1*, *ura3-1*). YJW 509, the *[psi$^-$] [pin$^-$]* derivative of this strain, was obtained by growth of YJW 508 on medium containing 5mM guanidine hydrochloride (Tuite et al., 1981). A *MAT**a** [psi$^-$] [PIN$^+$]* but otherwise isogenic strain (YJW 564) was obtained by transient over-expression of Hsp104p in a sister spore of YJW 508. The *[PSI$^+$] [pin$^-$]* derivative (YJW 616) was obtained as described in Figure 1A. Unless otherwise noted, all yeast methods were as described in (Sherman, 1991). Deletions of *HSP104*, *NEW1* and *RNQ1* were made using heterologous gene replacement (Longtine et al., 1998). *SUP35* was disrupted by omega integration of a linear DNA fragment consisting of the *TRP1* open reading frame flanked by the 500 base pairs on either side of *SUP35*.

Plasmid Construction

High copy yeast plasmids were derived from pRS424, pRS425 and pRS426, while low copy plasmids were derived from pRS314 and pRS315 (Christianson et al., 1992). All yeast expression vectors used a previously described modular system (Santoso et al., 2000) composed of a promoter flanked by 5' XhoI and 3' BamHI/SalI sites, a Gln/Asn-rich domain module flanked by 5' BamHI/SalI and 3' BglII/EcoRI sites and in-frame GFP/CFP/YFP/EF modules flanked by 5' BglII/EcoRI and 3' SacI/NotI sites.

Sup35p_{CA}-GFP, New-GFP and New-EF constructs were described previously (Santoso et al., 2000); for two color fluorescence experiments, the GFP modules of these constructs were replaced by the appropriate GFP variant (Clontech). Other Gln/Asn-rich domains were amplified by PCR from yeast genomic DNA using oligonucleotides with 5' BamHI or SalI and 3' EcoRI linkers and cloned into the above vectors.

Construction of polyGln-expanded MJD fragment: to avoid genetic instability associated with pure CAG repeats, we made designed complementary oligonucleotides encoding multiples of 20 glutamine residues using a mixture of CAG and CAA codons. These were annealed and inserted into the KpnI and BamHI sites of a bacterial shuttle vector. Inserting additional such cassettes into the

resulting construct increased the polyGln tract in increments of 20. The polyGln tracts were inserted after residue 14 of a truncated MJD fragment (residues 203-284) (Ikeda et al., 1996), which was then introduced into the GFP expression system described above.

High copy expression plasmids bearing *HSP104*, *SSA1* and *SSB1* were constructed by amplification from genomic DNA of these ORFS, as well as 500 bp on either side, followed by cloning into pRS423. Truncation constructs of New1p were generated by amplification of the DNA sequences corresponding to the indicated codons flanked by 5' BamHI and 3' EcoRI sites followed by cloning into the modular GFP vectors described above. For constructs missing the natural initiator codon, ATG was introduced prior to first indicated codon. The EE tag (Feschenko et al., 1992) was introduced by annealing two complementary oligonucleotides flanked by EcoRI and SacI sites and inserting them in place of GFP. New-GFP RR point mutants were generated using the QuickChange method (Stratagene).

Prion induction assays

Gene expression from high copy plasmids was induced with 50 μM $CuSO_4$ for ~48 hours. Serial dilutions were plated onto synthetic medium lacking adenine (-ade) and rich synthetic medium. In all

cases except in the D*new1* strain (as noted in Table 1), ADE+ colonies were counted after 5 days of growth. To generate *[NU⁺]* strains, we replaced the plasmid-borne *SUP35* gene in *[pin⁻]* and *[PIN⁺]* derivates of a D*sup35* strain with a low copy plasmid expressing New–EF from the *SUP35* promoter, then transiently over-expressed New-GFP and selected for ADE+ colonies as above.

Cytoduction

A *[nu⁻] [PIN⁺] [rho⁻] mata kar1*-D*13 sup35::TRP1 cyh2* pRS315-NEW-EF recipient strain was grown to log phase, mixed equally with similar cultures of *[nu⁻]* or *[NU⁺] [PIN⁺] [RHO⁺] mata KAR1 sup35::TRP1 CYH2* pRS315-NEW-EF donor strains and spotted onto YEPD plates. After 8 hours, the spots were streaked or patched onto YEPGlycerol with 10 *m*g/ml cyclohexemide, on which only *[RHO⁺] cyh2* cytoductants can grow (Rose and Fink, 1987). Patches and individual colonies from each cross were tested for ADE+.

Fluorescent microscopy and in vivo GFP foci formation assays

Yeast treated as in the prion induction assays above were examined by fluorescent microscopy (Zeiss Axiovert S100) through appropriate filters (Chroma) and photographed by CCD camera

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(Diagnostic Instruments Model 1.3.0). In aggregate counting experiments, at least five random fields with ~25-80 cells were examined by bright field microscopy, then by fluorescent microscopy; ratios reported indicate fraction of total cells displaying foci. Each experiment was performed at least 3 times; reported values are cumulative.

Other assays

Centrifugation was performed as described previously (DePace et al., 1998; Patino et al., 1996), using ~100 ml final lysate volumes. MJD-GFP centrifugation was performed with the addition of 1% Triton X-100 to the lysis buffer. Fractions were re-suspended in SDS-PAGE loading buffer with 4M urea. For immunoblotting, polyclonal rabbit a-Sup35p (Santoso et al., 2000) and monoclonal a-GFP (Roche) sera were used.

Acknowledgments

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Chapter IV. Dissection of New1p Prion Behavior

Abstract

The prion-forming properties of New1p arise from glutamine- and asparagine- rich (Q/N-rich) sequences near their amino terminus. We truncated and rearranged the various sequence elements within the New1p prion domain in order to dissect their contribution to prion induction and propagation. New1p prion induction requires a NYN repeat motif but stable maintenance of this prion state requires both the NYN motif and an oligopeptide sequence shared with Sup35p, another prion-forming protein. The NYN motif determines the specificity of New1p-derived prions and renders prions insensitive to the curing of effect of excess Hsp104p.

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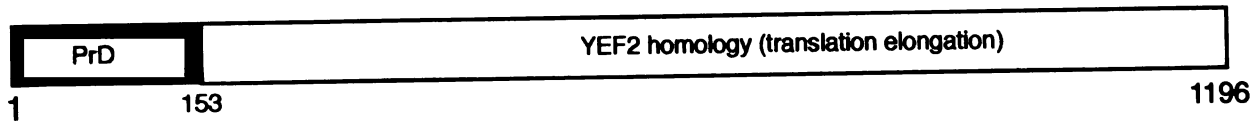
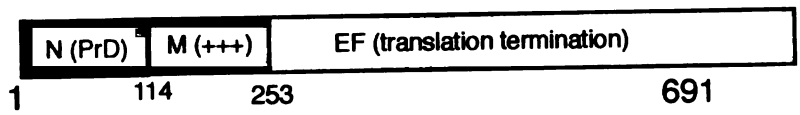
Introduction

Based on its genomic open reading frame, New1p is predicted to consist of two readily discernable domains; the amino-terminal portion, which is rich in glutamine and asparagine (Q/N) residues, and extended carboxy-terminal domain that is homologous to the translation elongation factor YEF2 (Figure 1A). We originally identified New1p on the basis of its homology to the prion-determining region of Sup35p, a protein with a similar domain structure (Figure 1B) (Santoso et al., 2000) (Michelitsch and Weissman, 2000). The regions of homology between the Q/N-rich regions of the two proteins are depicted in Figure 1C. Notable features of New1p include the repetitive NYN motif tract located between residues 71 and 100, as well as the oligopeptide sequence QQGGYQSYN, found between residues 61 and 69. The initial prediction of homology between New1p and Sup35p relied on the nearly perfect correspondence of this oligopeptide sequence with the degenerate family of oligopeptides found between residues 40 and 114 of Sup35p. Whereas in Sup35p the Q/N-rich region lies ahead of the oligopeptide region (encompassing residues 1- 38), in New1p, the oligopeptide precedes the NYN tract.

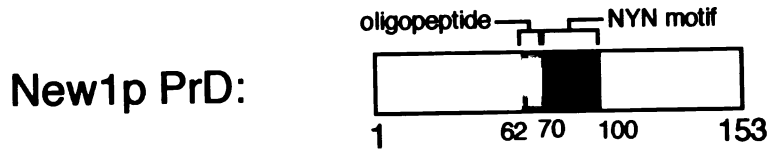
A


New1p
PrD
YEF2 homology (translation elongation)
1
153
1196

B


Sup35p
N (PrD)
M (+++)
EF (translation termination)
1
114
253
691

C


oligopeptide
NYN motif
New1p PrD:
1
62
70
100
153

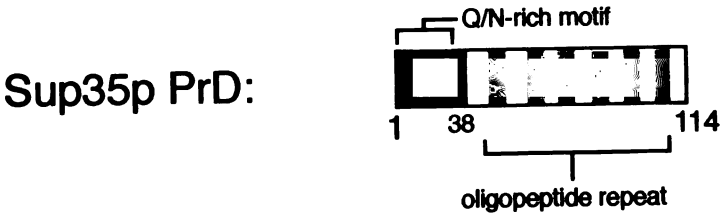

Q/N-rich motif
Sup35p PrD:
1
38
114
oligopeptide repeat

Figure 1. Schematic diagrams of New1p and Sup35p.

Numbers indicate amino acid positions.

(A) The domains of New1p. The prion domain (PrD) is the Q/N-rich region predicted by computational (Michelitsch and Weissman, 2000) and functional analysis (Santoso et al., Osherovich and Weissman, 2001). The YEF2 homology domain participates in translation termination (Osherovich and Weissman, unpublished data); see chapter 5.

(B) The domains of Sup35p. The prion domain (PrD) is needed for [*PSI*$^+$] induction and propagation, but is dispensable for translation-related activity. The lysine-rich M domain has no known function, while the C-terminal EF domain belongs to the eRF3 family of translation termination factors, and is essential for viability

(C) Comparison of New1p and Sup35p prion domains. The gray regions are degenerate oligopeptides with the consensus sequence QQGGYQQxYN; this sequence appears once in New1p and six times in Sup35p, and is the primary basis of homology between the two proteins. The Q/N-rich and NYN motifs are required for the aggregation of Sup35p and New1p, respectively, and contain an extraordinary density of glutamine and asparagine residues.

Mutational analysis of SUP35 has revealed a critical role for glutamine and asparagine residues near the amino terminus

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of the protein in maintenance and propagation of the [*PSI*$^+$] prion state. Deletion of the N domain of Sup35p (residues 1-113) abolishes the propagation and induction of [*PSI*$^+$] (Ter-Avanesyan et al., 1994), as do point mutations that alter the Q/N composition of the first 38 residues (DePace et al., 1998). The prion domain of Sup35p is modular, and, when transplanted to other proteins, leads to their reversible inactivation through prion formation (Patino et al., 1996) (Li and Lindquist, 2000). Both *in vivo* and *in vitro* studies have revealed that the Q/N-rich tract of Sup35p is critical for the aggregation of the protein (DePace et al., 1998). The adjacent oligopeptide region, however, has also been implicated in prion behavior Sup35p. Specifically, expansion of the oligopeptide tract results in enhanced spontaneous appearance of [*PSI*$^+$] (Liu and Lindquist, 1999), while the elimination of certain oligopeptide repeats prevents normal prion behavior by Sup35p (Parham et al., 2001). It thus appears likely that prion behavior of Sup35p involved contributions from both the Q/N-rich tract and the oligopeptide repeat region, although the precise nature of these contributions is unknown. One or more critical steps in the replication cycle of [*PSI*$^+$] involves the action of the chaperone Hsp104p, which is required for normal maintenance of this

prion; decreases or increases in the expression of Hsp104p lead to loss of the [*PSI*$^+$] prion state (Chernoff et al., 1995).

Our previous work has demonstrated that the prion domain (PrD) of New1p may be transplanted onto Sup35p to replace its own PrD, resulting in a chimerical protein, termed New-EF, that can interconvert between stably heritable soluble and aggregated states, termed [*nu*$^-$] and [*NU*$^+$] respectively (Santoso et al., 2000) (Osherovich and Weissman, 2001). Analogously to [*PSI*$^+$], the [*NU*$^+$] form of New-EF depletes the cell of essential Sup35p EF domain activity, causing a translation termination defect manifested by adenine prototrophy and pink colony color in the context of the *ade1-14* nonsense mutation. [*NU*$^+$] displays all the hallmarks of a yeast prion, including phenotypic variation (prion "strains"), cytoplasmic inheritance and dependence on Hsp104p for maintenance (Osherovich and Weissman, 2001).

We have taken advantage of the similarities and differences between New1p and Sup35p to probe the role of various subdomains of these proteins in prion behavior. Below, we present evidence that the oligopeptide and Q/N-rich regions play distinct roles in induction, propagation and specificity of New1p and Sup35p prions. The modular nature of these subdomains is demonstrated by the construction of a prion-

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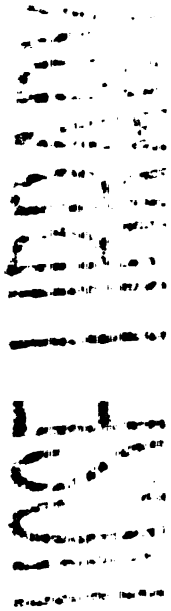
forming chimerical protein consisting of subdomains of both New1p and Sup35p.

Results

In order to further characterize the prion-forming properties of New1p, we first addressed the problem of high spontaneous conversion from [*nu*$^-$] to [*NU*$^+$] by the previously described New-EF fusion protein (Santoso et al., 2000). We observed that although the rate of appearance of adenine prototrophy in [*nu*$^-$] strains was stimulated by over-expression of the New1p prion domain (residues 1-153) fused to GFP, there was nonetheless an appreciable number of ADE+ convertants in control experiments without induction or with an empty vector in lieu of the inducer. Earlier reports on Sup35p had suggested that the middle (M) domain of this protein, located between the N and EF domains, renders the protein more soluble *in vitro* and less prone to spontaneous aggregation *in vivo* (Ter-Avanesyan et al., 1993) (Glover et al., 1997). We therefore introduced the M domain into the New-EF construct, between the New1p prion domain and the Sup35p-derived EF domain. When introduced into strains lacking endogenous Sup35p, the resulting chimerical protein, termed New-MEF, had a markedly decreased rate of



spontaneous aggregation compared with New-EF (Figure 2A), and was subsequently used in the experiments described below. As this fusion protein could interconvert between heritably soluble and aggregated forms, we retained the [*nu*$^-$] and [*NU*$^+$] nomenclature previously used in connection to New-EF fusion to describe these states.

The NYN tract of New1p has previously been implicated in both the ability of New1p to aggregate and its ability to stimulate Sup35p prion conversion and poly-Gln aggregation (Osherovich and Weissman, 2001). We subsequently examined the role of the NYN tract and other portions of New1p in the induction of [*NU*$^+$]. A series of truncations of New1p fused to GFP was expressed from a high-copy, copper-inducible vector in a [*nu*$^-$] strain bearing a low-copy constitutively expressed version of New-MEF. As shown in Figure 2B, the NYN tract of New1p is necessary and sufficient for induction; the appearance of ADE+ colonies was stimulated equivalently by GFP fusions of New1p residues 1-153 or residues 70-100, but not by constructs lacking the residues 70-100. Residues 70-100, which are

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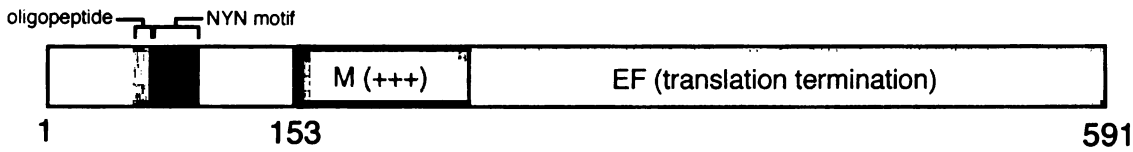
A



B

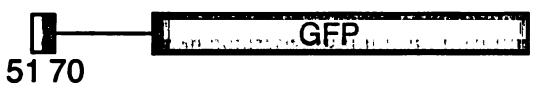
inducer (over-expressed)	*[NU⁺]* induction
vector control	No
	Yes
	Yes
	Yes
	No
	Yes

Figure 2. The NYN motif is necessary and sufficient for [*NU*$^{+}$] induction.

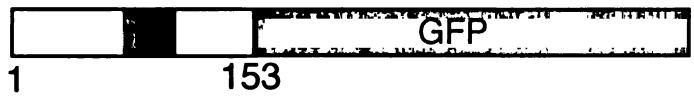
(A) Schematic diagram of improved reporter for New1p prion formation. This construct, termed New-MEF, incorporates the Sup35p M domain between a New1p-derived amino terminus and the translation domain of Sup35p. This construct was introduced on a low-copy vector and driven by the natural Sup35p promoter.

(B) Ability of various truncations of the New1p PrD to induce conversion of New-MEF to a stable prion form, [*NU+*]. Strains expressing New-MEF in lieu of endogenous Sup35p were transformed with high-copy, copper-inducible plasmids expressing the indicated GFP fusion protein. [*NU+*] induction was performed as described previously; in cases where conversion occurred, approximately 10% of plated cells acquired the ADE+ trait. Individual ADE+ clones were re-streaked and assayed for stability of ADE+ to confirm genuine conversion to [*NU+*].

needed for New1p to form aggregates when over-expressed, thus appear to be sufficient for both the induction of [*NU*$^+$] and the aggregation-promoting effect of New1p on other Q/N-rich proteins.

We next asked what portions of New1p were needed to maintain the [*NU*$^+$] prion state. We constructed a series of truncations of the New1p-derived portion of New-MEF and introduced these constructs on plasmids to replace the endogenous SUP35. We then over-expressed the New-GFP inducer and assayed for conversion of the truncated New-MEF chimera to an aggregated [*NU*$^+$] form (Figure 3). Consistently with its role in aggregation, we observed that the NYN tract is necessary for maintenance of the prion state. However, we also observed a requirement for the oligopeptide tract (residues 50-69) for proper maintenance of [*NU*$^+$], as constructs lacking this region could not be converted to a stable ADE+ state. Based on these data, we constructed a minimized version of New-MEF, termed mini-NEW, which consists of residues 50 through 100 of New1p fused the M and EF regions of Sup35p. Remarkably, this diminutive protein displayed all of the characteristics of the larger New-MEF constructs, including prion strain variability and reversible curing by guanidine hydrochloride (Figure 4).

inducer (over-expressed)



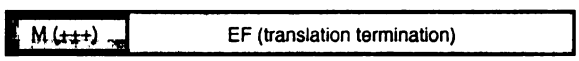
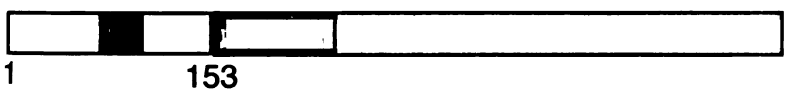
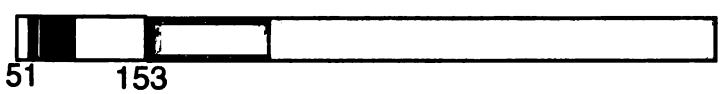
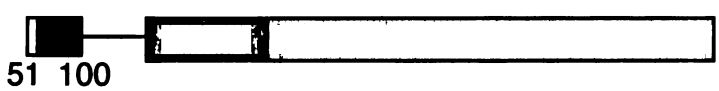
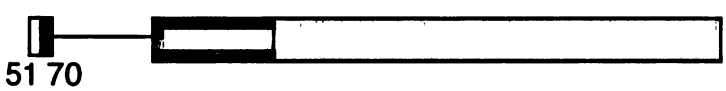
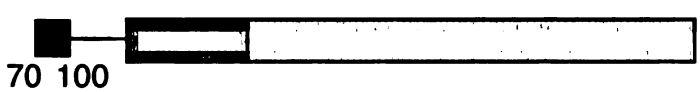
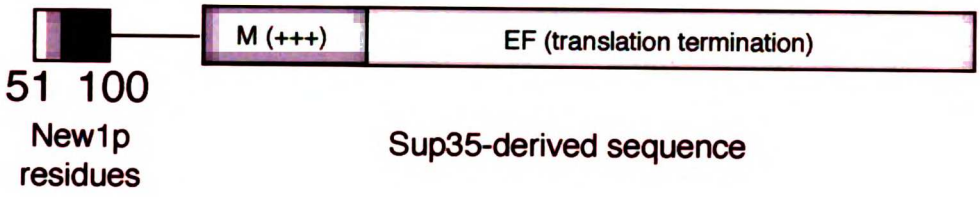
maintainer (constitutive)	conversion to [*NU*+]
	No
	Yes
	Yes
	Yes
	No
	No

Figure 3. Both the NYN motif and the oligopeptide region of New1p are required for maintenance of the [*NU+*] prion state.

In these experiments, New1p residues 1-153 fused to GFP (inducer, shown above) was over-expressed in strains bearing the indicated truncation of New-MEF (maintainer) in lieu of endogenous Sup35p. [*NU+*] induction assay was performed as described in Figure 2.

A

mini-NEW



B

YEPD low ADE

SD no ADE

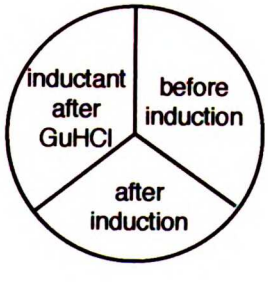


Figure 4. Minimization of New1p prion domain

(A) The smallest New1p-derived construct capable of maintaining the [*NU+*] state, termed mini-NEW.

(B) Mini-New forms a reversible prion state identical to the [*NU+*] state of the larger NEW-MEF protein. Shown are streaks of SUP35-deleted strains bearing mini-NEW prior to transient over-expression of New-GFP, after selection on SD-A and loss of the New-GFP inducer plasmid, and after growth of these ADE+ derivatives on medium containing 5mM guanidine hydrochloride. The pink color of the [*NU+*] derivative on YEPD medium (left) corresponds to adenine prototrophy (right). The isolate shown is [*pin*$^{-}$], demonstrating the independence of mini-New prions on other factors; [*PIN*$^{+}$] derivates were also obtained (data not shown).

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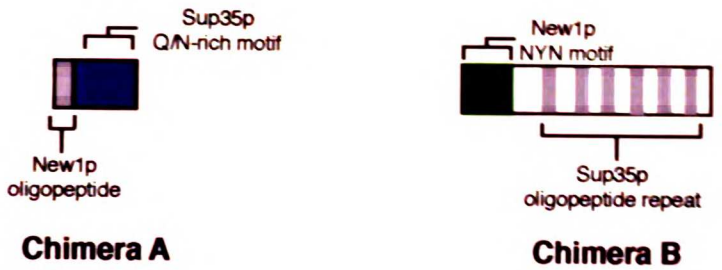
The preceding experiments suggested that both a Q/N-rich tract and an oligorepeat sequence are needed for proper prion behavior. We thus examined how alteration of the composition and arrangement of these sequence elements affected prion behavior. We constructed chimerical fusion proteins with swapped sequence elements from New1p and Sup35p and assayed their ability to induce [*NU+*] or [*PSI*$^+$] as well as their ability to themselves adopt a prion state (Figure 5). One chimera, consisting of New1p residues 70-100 and Sup35p residues 40-114, efficiently induced the [*NU+*] state when over-expressed as a GFP fusion, but did not promote the appearance of [*PSI*$^+$] prions. Furthermore, a fusion of this chimera to the MEF portion of Sup35p resulted in a prion-forming protein that displayed the characteristics of [*NU+*], including preferential induction by New1p over-expression and lack of dependence on the [*PIN*$^+$] element for efficient induction (see chapter 3). In contrast, the reciprocal chimera consisting of residues 1-38 of Sup35p and residues 50-69 of New1p could neither induce the appearance of [*NU+*] or [*PSI*$^+$] prions nor support the propagation of a prion state after over-expression of New1p or Sup35p.

A

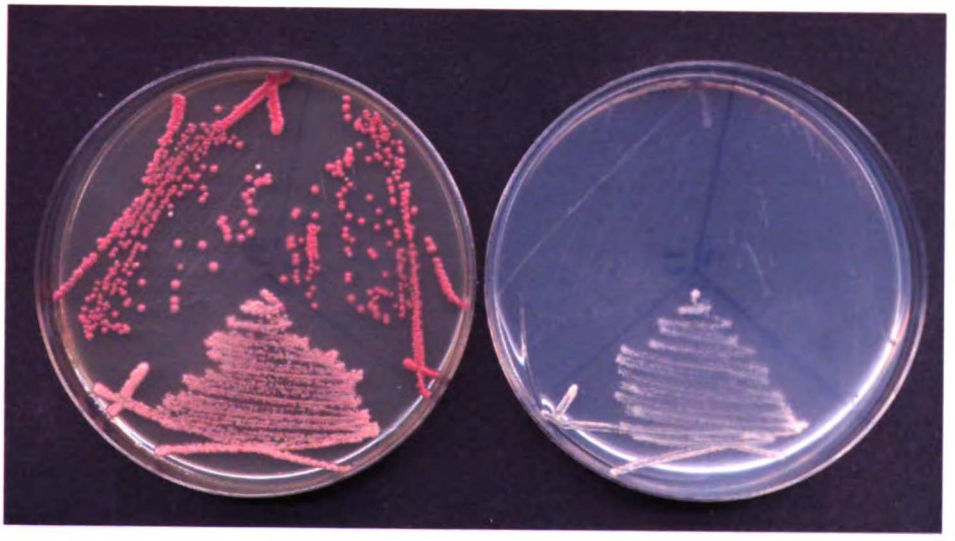


Chimera A **Chimera B**

B

maintainer	inducer: vector	SUP-GFP	NEW-GFP	Chimera A - GFP	Chimera B - GFP
SUP-MEF	-	+++	-	-	-
NEW-MEF	-	-	+++	-	+++
Chimera A MEF	-	-	-	not tested	not tested
Chimera B MEF	+	+	+++	not tested	+++

C



YEPD low ADE SD no ADE

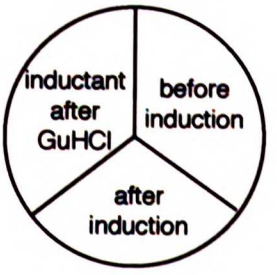


Figure 5. Prion properties of Sup35p-New1p PrD chimeras

(A) Schematic diagrams of two complementary chimerical fusions between the prion domains of Sup35p and New1p. Chimera A consists of New1p residues 51-70 and Sup35p residues 2-38, while chimera B consists of New1p residues 71-100 and Sup35p residues 40-114.

(B) Prion formation and specificity of chimerical PrD fusions. High copy, copper-inducible GFP fusions (inducers) of indicated proteins were expressed in strains bearing the indicated low copy MEF fusions (maintainers). – indicates no ADE+ colonies, +++ indicates extensive conversion (~10%), and + indicates low frequency conversion (~0.5%). Chimera A was incapable of inducing or maintaining prion forms of any protein, whereas chimera B could convert New-MEF to a [*NU+*] state and, as a maintainer, could be converted by New-GFP or Chimera B–GFP over-expression. The Chimera B-MEF maintainer had a higher spontaneous rate of conversion to an inactive state, as seen by the appearance of a small number of ADE+ colonies in vector-only or Sup-GFP inducer experiments; when re-streaked, these strains rapidly reverted to ade-, suggesting that these were not *bone fide* prion convertants. The prion specificity of stable ADE+ isolates was assayed by galactose-induced expression of an additional maintainer with either a Sup35p or New1p prion domain. The expression of Sup-MEF

abolished adenine protrophy (antisuppression) while New-MEF expression did not alter adenine protophy, indicating that chimera B ADE+ isolates were [*NU+*] (data not shown).

(C) Prion basis of chimera B ADE+ trait. Isolates are shown before and after induction of ADE+ by New-GFP over-expression. ADE+ could be eliminated by guanidine hydrochloride treatment.

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Finally, we examined the effect of altering chaperone levels on the propagation of the [*NU+*] states. Hsp104p, a chaperone required for the maintenance of all known prion states, had previously been shown to antagonize propagation of [*PSI*$^+$] (Chernoff et al., 1995). We observed that over-expression of Hsp104p had no effect on [*NU+*], whereas this treatment lead to the efficient loss of [*PSI*$^+$] in otherwise isogenic strains (Figure 6). The lack of curing of [*NU+*] by Hsp104p over-expression was observed with both weak and strong prion "strains," indicating this effect was not simply due to more pervasive aggregation by New1p compared to Sup35p. As the prion form of the mini-NEW protein was also refractory to Hsp104p over-expression, the differential sensitivity of the two types of prions could not be explained by the presence of antagonistic elements outside of the central prion-forming region of New1p. We found that the prion form of the New1p$_{70-100}$-Sup35p$_{40-114}$ chimera was also resistant to the curing effects of Hsp104p over-expression, despite being mostly derived from Sup35p (data not shown). Additionally, a mutant form of New1p containing an expansion of the oligopeptide repeat sequence to a number near to that found in Sup35p (five in total) could adopt a [*NU+*]-specific prion state, but could not be cured by Hsp104p

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over-expression. Thus, the aggregation-determining NYN tract of New1p appears to render prions that contain it resistant to the action of Hsp104p over-expression.

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low ADE

no ADE

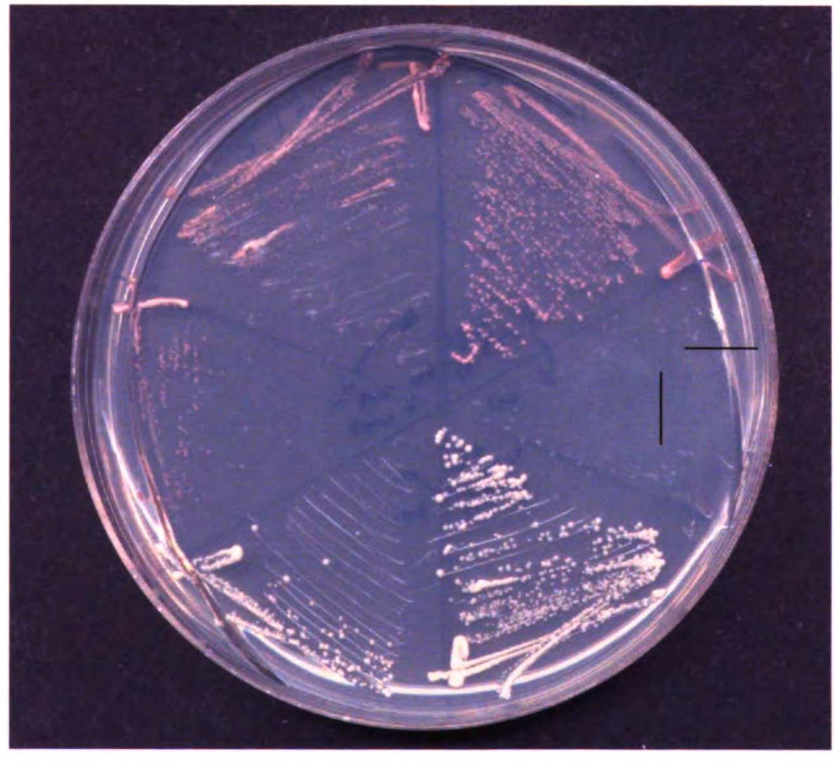

Figure 6. Hsp104p over-expression does not eliminate [*NU+*].

Strains bearing the indicated prion were transformed with galactose-inducible plasmids encoding Hsp104p or control vectors and grown on SGR (inducing) medium. As previously reported, Hsp104p over-expression eliminates [*PSI*$^+$], resulting in a red colony color on low adenine medium and lack of growth on adenine-free medium. Neither full length [*NU+*] (New1p residues 1-153) nor mini-[*NU+*] (residues 51-100) were affected by Hsp104p over-expression. The lack of Hsp104p curing of [*NU*$^+$] is not due to elevated levels of New-MEF or mini-New proteins as a result of multiple plasmid copies, as the [*PSI*$^+$] control shown here also has a plasmid-encoded Sup35p.

Discussion

We have dissected the sequence and structural elements of two prion-forming proteins, New1p and Sup35p and have uncovered distinct regions within these proteins that facilitate different aspects of the prion replication cycle. Because of their modular nature, the prion domains of Sup35p and New1p are amenable to minimization and sequence swapping experiments. These techniques have allowed us to identify functionally similar portions of the two proteins and to analyze the contribution of these subdomains to aggregation specificity, stable inheritance and interactions with chaperones.

Our most significant finding is that the two aspects of prion behavior, initial aggregation and maintenance of the aggregated state, arise from distinct portions of New1p and can be uncoupled. The NYN tract of New1p, which is required for New1p aggregation and promotes the susceptibility to aggregation of other Q/N-rich proteins, is necessary but not sufficient for prion behavior by New1p. We found that, when over-expressed, the NYN tract could readily aggregate and recruit larger New1p fragments into a prion state. However, the NYN tract could not itself support stable prion inheritance,

which required an additional sequence element, the oligopeptide repeat that is common to both New1p and Sup35p. Remarkably, the lengthy oligopeptide region of Sup35p could functionally replace that of New1p, resulting in a prion-forming protein with the majority of sequence derived from Sup35p but with the prion characterisitics of New1p.

Although Sup35p and New1p prion domains contain similar Q/N-rich and oligopeptide regions, the specific sequence, size and orientations of these subdomains differ. Our minimization and substitution experiments hint at how these differences affect the functions of these prion domains. Glutamines and asparagines in the first 38 residues of Sup35p are required for the aggregation of Sup35p and dictate the specificity of homotypic prion interactions. We have shown that the first 38 residues of Sup35p can be replaced with the NYN tract of New1p to create a chimerical protein that forms a prion state that is indistinguishable from that of New1p. This finding indicates that, although the remaining regions of Sup35p are important for prion propagation, the specificity of this prion state can be largely determined by a short, Q/N-rich sequence. Unlike the previously described chimera between *Candida albicans* and *Saccharomyces cerevisiae* Sup35p prion domains (Chien and Weissman, 2001), this Sup35p-New1p fusion does

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not display dual specificity and is instead overwhelmingly disposed to adopting a New1p-specific state.

In New1p, the oligopeptide region is smaller than that of Sup35p, with one complete oligopeptide (QQGGYSYN) preceded by a degenerate sequence (QGGNY) reminiscent of a partial oligorepeat. Earlier reports have demonstrated the importance of the Sup35p oligorepeats in [*PSI*$^+$] propagation (Liu and Lindquist, 1999) (Parham et al., 2001), and their conservation among Sup35p homologs from multiple yeast species suggests an adaptive function (Santoso et al., 2000) (Chernoff et al., 2000) (Kushnirov et al., 2000). We have demonstrated that, as in Sup35p, the oligopeptide portion of New1p is required for prion maintenance. However, this region is not needed for *de novo* aggregation of New1p or the induction of the [*NU+*] state, suggesting that it participates in a distinct step in prion replication that occurs subsequently to aggregation. Oligopeptide sequences from either New1p or Sup35 can support prion replication by the New1p-derived NYN tract, while prion behavior of Sup35p residues 1-38 evidently requires the longer oligopeptide repeat sequence. However, the prion properties of the New1p$_{70\text{-}100}$-Sup35$_{40\text{-}114}$ chimera (Chimera B) indicate that for [*NU+*] prion formation, the position of the oligopeptide repeat can be switched from the N-terminal side of

the NYN to the C-terminal side without altering prion behavior or specificity.

The ability of the Sup35p oligopeptide sequence to substitute that of New1p suggests that oligopeptides act in a general manner to facilitate prion replication. One possible role for the oligopeptide sequence could be as an epitope for recognition by chaperones such as Hsp104p and Ssa1p, which are thought to regenerate prion seeds to insure prion replication through cell division (Kushnirov and Ter-Avanesyan, 1998) (Jung et al., 2000). The failure of New1p-derived constructs lacking the oligopeptide region to maintain a prion state, despite being capable of forming aggregates, could result from the cell's inability to break these aggregates up into productive, self-regenerating prion seeds. Further experiments will address the possibility of specific chaperone interactions between the oligorepeat portions of Sup35p and various chaperones.

We have also attempted to address the mechanism of Hsp104p-mediated curing of [*PSI*$^+$]. We observed that the over-expression of Hsp104p could efficiently eliminate [*PSI*$^+$], but had no discernable effect on [*NU*$^+$]. To identify the portion of Sup35p responsible for this curing effect, we assayed the curability of prion forms of chimerical constructs incorporating elements of New1p and Sup35p. We observed that [*NU+*] specificity, which

arose from the presence of the NYN motif in the chimeric protein, always correlated with the lack of curing by Hsp104p over-expression, even in the case of Chimera B, which consists mostly of Sup35p-derived sequences. One explanation for these findings is that Hsp104p recognizes the very same region of Sup35p that is involved in [*PSI*$^+$] specificity (residues 1-38). Alternatively, the curing effect of excess Hsp104p on [*PSI*$^+$] may be an idiosyncrasy of this prion's aggregated conformation, which may differ from that of [*NU+*] or other prions in a way that renders it accessible to Hsp104p. This latter model may also involve differences in the extent of aggregation between Sup35p and New1p; for instance, NYN motif-containing prions may be too extensively aggregated for Hsp104p to have any effect. Finally, it is possible that [*PSI*$^+$] curing effect of excess Hsp104p may be an indirect consequence of alterations in the activity of Ssa1p, an Hsp70 protein needed for the replication of [*PSI*$^+$] but not other prions [Newnam, 1999 #46] (Jung et al., 2000). To examine these possibilities, we are currently performing a genetic screen for mutants of Sup35p that, when in a [*PSI*$^+$] state, are insensitive the curing effect of over-expressed Hsp104p.

Experimental Procedures

Yeast Strains and Methods

Strains were derived from the W303 background, as described previously, and standard yeast methods were followed (Sherman, 1991). Unless otherwise noted, strains were [*PIN*$^+$]. To introduce the described MEF constructs, we transformed strains with the genotype [*psi*$^-$] [*PIN*$^+$] Matα *sup35::*TRP1 *ade1-14 his3-11,15 leu2-3 trp1-1 ura3-1* pRS315SpSUPMEF with URA-marked (pRS316-derived) plasmids bearing the indicated gene, selecting for loss of leucine prototrophy . Hsp104p induction assays were performed on synthetic defined (S) medium containing 2% galactose and 2% raffinose.

Plasmid Construction

We utilized the modular cloning system described for SUP35 and NEW1 previously. Plasmid vectors used were derived from (Sikorski and Hieter, 1989). The improved New-MEF construct was produced by replacement of the EF portion of the previously described New-EF with PCR-amplifed DNA consisting of the MEF portion of SUP35 flanked by BglII and SacI sites. Truncations of the New1p prion domain were derived from those described in (Osherovich and Weissman, 2001); here, the prion

domains were subcloned as BamHI/EcoRI fragments upstream of MEF. Chimera A was produced by subcloning a BamHI-New1 $_{51-69}$-EcoRI fragment upstream of a Sup35$_{2-38}$-MEF construct. Chimera B was the product of insertion of a BamHI-New1$_{70-100}$-EcoRI fragment upstream of a Sup35$_{40-114}$-MEF construct. In all cases, initiator ATG codons were introduced if not already present. Galactose-inducible HSP104 was made by cloning of BamHI-HSP104-SacI downstream of a Gal1 promoter introduced into pRS315.

Prion induction assays

Strains were transformed with high-copy (2 micron) plasmids expressing the GFP fusion protein in question from a copper-inducible promoter and treated as described previously (Osherovich and Weissman, 2001). Conversion to a prion state was assessed by stable adenine prototrophy that remained after loss of the inducing plasmid that could be eliminated by transient growth on medium containing 5mM guanidine hydrochloride.

Chapter V. It Didn't Work!

Introduction

Good science raises more questions than it answers. At the outset of my doctoral studies, our understanding of yeast prion behavior was quite naïve in comparison with what we know today. During my five years at the bench, there has been a great deal of progress in our field as a whole, let alone in the specific areas on which I have focused my work. Nonetheless, we must acknowledge that we know very little about how proteins misfold, why certain misfolded proteins form aggregates, why certain of these aggregates are self-propagating, why certain of these self-propagating aggregated states are stably inherited from cell to cell and, in higher organisms, why such conditions can sometimes lead to disease and death. In this chapter, I will highlight what I consider to be the most important unanswered questions in arising from my work. My intention is to summarize the ideas and experiments that I've tried out already in order to help future researchers to choose reasonable projects.

The Mechanism of [*PIN*$^+$]

The most compelling and elusive question arising from my doctoral work is the mechanism by which the presence of certain prions predisposes other glutamine- and asparagine-rich (Q/N-rich) proteins to aggregate. In 1997, Derkatch et al. demonstrated a striking and mysterious phenomenon; certain strains could readily undergo conversion from [*psi*$^-$] to [*PSI*$^+$] upon over-expression of Sup35p, but this prion inducibility could be abolished by transient growth on medium containing low concentrations of guanidine hydrochloride (Derkatch et al., 1997). Subsequent work from that laboratory demonstrated that this phenomenon was due to a cryptic prion, but its identity, much less its mode of action, was unknown (Derkatch et al., 2000). My own contribution to this area, performed in remarkable and fortuitous synchrony with Derkatch et al. (2001), helped to identify the responsible proteins, but only deepened the mystery of the mechanism (Osherovich and Weissman, 2001).

The state of the field is the following: aggregates of a number of different Q/N-rich protein, including New1p and Rnq1p, act in a dominant manner to facilitate the aggregation of other Q/N-rich proteins (Sup35p and transgenic human polyGln disease proteins such MJD and huntingtin) that ordinarily unable to aggregate. In Chapter 1 of this thesis, I discussed the three major models that we

have proposed to explain this phenomenon, but despite a year of effort, I have not made any substantive advances toward proving or disproving any of these models.

Candidate Approach: the Proteasome

The model on which I have focused most of my effort is referred to as inhibition of anti-aggregation (see Chapter 1). To recapitulate, this model predicts that aggregates of Rnq1p or New1p bind to and inactivate one or more factors that ordinarily function to prevent the aggregation of Q/N-rich proteins. To test this model, I have used a candidate approach, focusing components of the ubiquitination and proteasome degradation pathway. I chose the proteasome as the most likely candidate for inactivation by [*PIN*$^+$] and [*NU*$^+$] prions based on published reports of inhibition of the mammalian proteasome by polyGln aggregates(Bence et al., 2001), my own observation of a potentially proteolytic variant of Sup35p that was influenced by [*PIN*$^+$] (see below), and, finally, by rumors in the yeast prion community that ubiquitination mutants displayed defective prion propagation. Below I outline what I tried to do; I include technical details in order to assist anyone attempting to repeat my experiment, and to help future researchers to figure out

what I did wrong in case the target of [*PIN*$^+$] really does turn out to be the proteasome.

According to one model, what makes a cell resistant to prion induction ([*pin*$^-$]) is the rapid degradation of misfolded Sup35p before it has a chance to aggregate. Thus inhibition of proteasome activity might facilitate prion conversion. To test this idea, I attempted to pharmacologically inhibit the proteasome and to assay for susceptibility to [*PSI*$^+$] induction in a [*pin*$^-$] strain. The first step toward this was to make yeast strains permeable to the proteasome inhibitor MG132 (Lee and Goldberg, 1996). Following the literature on this topic, I generated an *erg6/ise1*-defective strain that was supposedly permeable to proteasome-inhibiting drugs due to a defect in cell wall biosynthesis (Gaber et al., 1989).[1] However, this mutant did not display the published sensitivity to MG132; both wild type and mutant strains had equivalent growth curves at high (200 μM) concentrations, and did not develop the reported thermotolerance associated with proteasomal inhibition (Lee and Goldberg, 1998).

I next turned to another genetic strategy for permeablizing yeast to MG132, generating strains defective in the multi-drug efflux

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[1] This proved rather difficult, as *ERG6* could not be disrupted in a tryptophan auxotrophic strain such as mine. This is a good lesson in the importance of reading primary literature before attempting an experiment.

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pump PDR5 (Emter et al., 2002) and the proteosomal transcriptional regulator RPN4 (Xie and Varshavsky, 2001). To test the effectiveness of MG132 treatment in these backgrounds, I introduced a degron-tagged GFP reporter (kindly provided by Neal Bence of the Kopito Laboratory) into *Δprd5, Δrpn4* and *Δprd5Δrpn4* strains and observed stabilization of GFP signal in the presence of MG132, indicating successful proteasomal inhibition. This fluorescence stabilization correlated with a decreased doubling time and a readily discernable septation defect in microscopically examined cells. I found that the *pdr5* mutation did not enhance MG132 sensitivity by itself or in conjunction with *rnp4*, so I subsequently worked with the *Δrnp4* single mutant.

To test the effect of proteasome inhibition on prion induction, I treated [*pin*$^-$] and [*PIN*$^+$] derivatives of the *Δrpn4* expressing either Sup-yCFP (Sup35p residues 1-253 fused to eukaryote-optimized cyan fluorescent protein) or a control vector with 200 μM MG132. After 15 and 40 hours at 30°, there was no difference between treated or untreated strains; [*pin*$^-$] strains displayed diffuse cytoplasmic fluorescence, while [*PIN*$^+$] strains displayed characteristic fluorescent aggregates and fibrils. There was a decreased rate of growth in the MG132-treated cultures compared to mock-treated controls, indicating that the drug was effective at disrupting proteasome function. After 40 hours, the cells were plated

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onto SD-A medium, but after five days of growth, there was no difference between treated and untreated cells; [*pin*$^-$] strains did not convert to [*PSI*$^+$], whereas [*PIN*$^+$] strains did.

A related strategy involved the genetic inactivation of the ubiquitination pathway, which targets substrates for degradation by the proteasome (Smith et al., 1996). My hope was that by altering the manner in which misfolded proteins are metabolized at this early point in the degradation pathway, I could prevent the degradation of Sup35p and increase its propensity to form prions, even in a [*pin*$^-$] strain. Although a staggering number of proteins participate in the addition of polyubiquitin chains to substrates destined for the cellular wastebasket, I chose the ubiquitin conjugating enzymes (E2) Ubc4p and Ubc5p as likely targets, as these two nearly identical homologs were reported to be responsible for the majority of degradation of misfolded proteins (Seufert and Jentsch, 1990). Furthermore, Chernoff's group had presented data to suggest that *ubc4* mutants had altered [*PSI*$^+$] behavior, although the specific effect was somewhat confusing.

I generated [*psi*$^-$] and [*PIN*$^+$] derivates of *Δubc4* and *Δubc5* mutants and attempted to cross them to produce a double mutant entirely deficient in misfolded protein-specific E2 activity. Despite published reports that this double mutant was viable, in my strain background, these mutations appeared to by synthetically lethal.

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Consequently, I tested the single mutants for altered prion induction, but observed no effect either way.[2] Because of the redundancy of the Ubc4p and Ubc5p proteins, I have not conclusively ruled out a role for these enzymes in prion induction, but as my attempts to make an inducible UBC5 construct were unsuccessful, I grew despondent and abandoned this line of experimentation.

Unbiased Approach: Genetics

Stepping back from directed guesswork, I next undertook genetic screens to tease out modifiers of prion induction. Derkatch had reported her extensive effort to identify genomic mutants that emulated [*PIN*$^+$] in an otherwise [*pin*$^-$] background; the failure to find such mutants lead her to favor the direct cross-seeding model for [*PIN*$^+$] action (Derkatch et al., 2001). I tried an alternative strategy: high copy suppression of [*PIN*$^+$]. A 2μ plasmid library ostensible derived from the Nasmyth library was transformed into [*PIN*$^+$] strains bearing an inducible Sup-GFP construct;

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[2] A curious observation: *ubc4* mutants are incapable of maintaining 2μ plasmids, making it necessary to use a CEN/ARS-based copper-inducible expression system in these experiments Sleep, D., Finnis, C., Turner, A., and Evans, L. (2001). Yeast 2 micron plasmid copy number is elevated by a mutation in the nuclear gene UBC4. Yeast *18*, 403-421..

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transformants were replica plated onto inducing (copper-containing) adenine-free medium. I screened approximately 10,000 transformants, which, given an average insert size of 6 kb and a total yeast genome of 6 x 10^3 kb, represents 10-fold coverage of the genome. Strains that failed to grow were re-streaked from the original plates, and the LEU2-marked plasmids were recovered by yeast mini-prep.

A higher than expected number of colonies passed the initial screen, so I chose six clones for further characterization. These plasmids retested positively in the starting strain and when re-transformed into fresh strains, but proved refractory to sequencing with Nasmyth library primers. Subsequent restriction digestion indicated that these plasmids were highly abnormal, and did not resemble known 2μ or CEN/ARS vectors; I have no explanation for what these plasmids are or where they came from. One clue about the activity of these plasmids came from microscopy; induced Sup-GFP in [*PIN*$^+$] strains bearing these plasmids displayed the characteristic pattern of aggregation, suggesting that these plasmids did not eliminate the [*PIN*$^+$] trait nor interfere with its manifestation at the level of *de novo* aggregation. It is possible that these plasmids prevent the manifestation of [*PSI*$^+$] by antagonizing read-through of the

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ade1-14 nonsense codon. As this scenario is less than interesting, I moved on.

Concurrently with the above-described work, I set up a selection to identify Sup35p mutants that could induce [*PSI*$^+$] in [*pin*$^-$] strains. Such a mutant had previously been obtained accidentally; Derkatch et al. (2000) reported a mutant form of Sup35p that terminates after the end of the N domain but bears a 17 amino acid C-terminal extension derived from the vector polylinker cloning site. As this protein was grossly altered from the normal form, the mechanistic basis of this [*PIN*$^+$] bypass was unclear. I reasoned that point mutations that permit the aggregation of Sup35p in a [*PIN*$^+$]-independent manner might point to the mechanism of interaction of Sup35p with cofactors during prion induction.

To obtain such a mutant, I amplified the DNA encoding the N and M regions of Sup35p using mutagenic (standard Taq) conditions, then transformed it along with a gapped high-copy copper-inducible GFP plasmid into [*psi*$^-$] [*pin*$^-$] yeast; the resulting recombinant plasmids ought to have expressed a mutagenized Sup-GFP protein when plated onto $CuSO_4$-containing medium. I screened a pool of ~2,250 mutants

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(representing full coverage of the mutagenized interval[3]) by scraping the transformation plates and plating ~100,000 colonies onto SD-A medium supplemented with 50 μM $CuSO_4$. 43 colonies came up after five days, and of these only seven turned red on medium containing GuHCl, which distinguishes the *bone fide* [*PSI*$^+$] convertants from weird random adenine prototrophs. Unfortunately, when these plasmids were recovered from the transformed strains and re-transformed into fresh [*psi*$^-$] [*pin*$^-$] strains, there was neither induction of Sup-GFP aggregation (as assayed by microscopy) nor of [*PSI*$^+$] (as assayed by adenine prototrophy). It thus appeared that the clones that I picked were spontaneous convertants to [*PSI*$^+$] that occurred in a plasmid-independent manner. Although it is higher than the reported background rate of one in ~10^6, this [*PIN*$^+$]-free conversion to [*PSI*$^+$] may have been enhanced by the adenine selection and growth conditions that I used. Despite this failure, it is nonetheless possible that a more systematic screen performed by some enterprising future student may yet

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[3] Taq polymerase makes 2.2 x 10^{-5} errors per nucleotide per round; after 30 rounds, an average of two of three 1 kb fragments (and transformants) will have one point mutation in them. In a pool of 2,250 transformants, the average nucleotide will thus have been mutagenized ~1.5 times. Greater coverage would have been better, however, but given the redundancy of the genetic code, this is adequate.

reveal Sup35p mutants that bypass the requirement for [*PIN*$^+$] in [*PSI*$^+$] induction.

Biochemical Approaches

An altogether different plan of attack on the [*PIN*$^+$] problem is to identify a biochemical activity responsible for inhibiting [*PSI*$^+$] induction in [*pin*$^-$] strains or promoting [*PSI*$^+$] induction in [*PIN*$^+$] strains. Although the biochemistry of the spontaneous and seeded conversion of recombinant Sup35p into amyloid fibrils has been extensively studied, the [*PIN*$^+$] phenomenon demonstrates that, *in vivo*, the process of prion induction and propagation undoubtedly involves the interaction of multiple cellular factors beyond Sup35p. I have attempted to develop an *ex vivo* assay for Sup35p conversion using extracts from [*pin*$^-$] and [*PIN*$^+$] strains, in hopes of observing different rates of seeding or spontaneous polymerization. The ultimate goal of such a project would be to biochemically fractionate factors influencing prion induction or propagation.

The *ex vivo* polymerization assay utilizes recombinant Sup35 NM protein that has been modified to include a cysteine residue coupled to a fluorescently labeled maleimide crosslinker (courteously provided by Dr. Helmut Sparrer). When this protein polymerizes, the fluorescent signal becomes quenched due to

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alterations in the chemical environment of the fluorophore. I prepared lysates of [*psi*$^-$] [*pin*$^-$], [*psi*$^-$] [*PIN*$^+$] and [*PSI*$^+$] [*PIN*$^+$] strains, added a small amount of labeled Sup35 NM monomer and monitored fluorescence over time. In several such experiments, it appeared that Sup35p polymerization was inhibited or delayed in [*psi*$^-$] extracts (both [*pin*$^-$] and [*PIN*$^+$]) compared to [*PSI*$^+$] extracts or extract-free buffer. A similar inhibitory activity had been previously reported by the Lindquist group, who had encountered difficulties in seeding of Sup35p by [*PSI*$^+$] extracts (Uptain et al., 2001). As fluorescently labeled protein was difficult to obtain in large quantities, I next attempted to reproduce this phenomenon with a thioflavin T dye-binding assay using non-labeled Sup35 NM protein. In this assay, fluorescent signal increases as the protein forms dye-binding amyloid fibrils (LeVine, 1999). Unfortunately, I did not obtain reliable results using this assay; my extracts appeared to inhibit thioflavin T binding or fluorescence. I subsequently abandoned this line of experimentation, but there is no reason why it shouldn't work if someone were willing to try harder than I did.

My initial enthusiasm for the proteasome hypothesis was in part informed by a biochemical observation. As discussed in Chapter 3, the expression of New1p causes [*pin*$^-$] strains to transiently assume a [*PIN*$^+$]-like state. I observed that in [*psi*$^-$] [*pin*$^-$] extracts, Sup-GFP migrated as a doublet, and that the relative distribution of

the two bands was influenced by the presence of excess New1p. As depicted in Figure 1A, antiserum against Sup35 NM recognizes two major bands in strains with over-expressed Sup-GFP (i.e. under conditions of [*PSI*$^+$] induction). The larger of the bands (~68 kD) is the full-length protein, while the smaller (~63 kD) lacks the extreme the amino terminal region (Figure 1B), suggesting that the smaller of the two bands is a proteolytic fragment of the larger one. As the larger band predominated in both [*psi*$^-$] [*PIN*$^+$] and [*PSI*$^+$] [*PIN*$^+$] extracts, it was unclear whether this accumulation was a cause or consequence of conversion to [*PSI*$^+$]; however, a mutant version of Sup-GFP that was incapable of aggregating (generously provided by Angela DePace) also appeared to accumulate in a New1p-over-expressing strain (Figure 1C), indicating that the basis for variation between the two bands was not aggregation. I subsequently spent a great deal of time characterizing this band shift in hopes of

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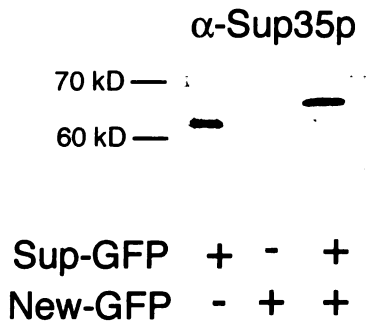
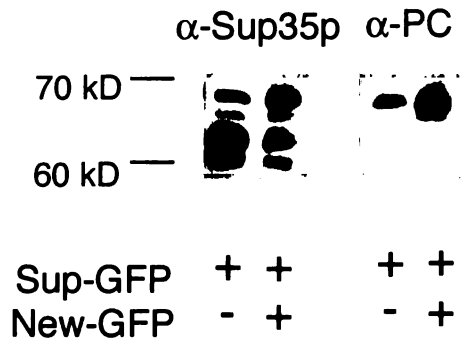
A


α-Sup35p
70 kD
60 kD
Sup-GFP + - +
New-GFP - + +

B


α-Sup35p α-PC
70 kD
60 kD
Sup-GFP + + + +
New-GFP - + - +

C

α-Sup35p
Asu-GFP + +
New-GFP - +

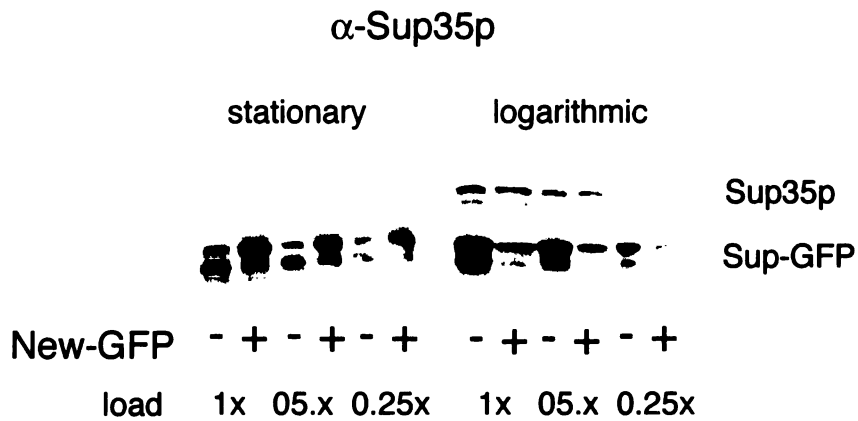

α-Sup35p
stationary
logarithmic
Sup35p
Sup-GFP
New-GFP - + - + - + - + - + - +
load 1x 05.x 0.25x 1x 05.x 0.25x

Figure 1. The size of Sup35p is influenced by New1p over-expression.

(A) Immunoblotting of [*psi*$^-$] [*pin*$^-$] extracts reveals that the presence of excess New-GFP results in decreased electrophoretic mobility of Sup-GFP. In [*psi*$^-$] [*PIN*$^+$] strains, there is a similar increase in the intensity of the larger band compared to [*pin*$^-$] strains.

(B) The smaller anti-Sup35p-reactive band is missing its extreme amino terminus. A version of Sup-GFP with an amino-terminal Protein C (PC) tag was expressed in [*psi*$^-$] [*pin*$^-$] cells with and without concomitant New-GFP over-expression, and immunoblotted with anti-Sup35p and anti-Protein C serum. The latter only recognizes the larger of the two bands.

(C) The aggregation-compromised Sup-GFP S17R mutant nonetheless displays a similar increase in large band intensity upon New-GFP over-expression.

(D) Two isoforms of full length Sup35p are observed in logarithmically growing cultures, whereas Sup35p levels are extremely low in stationary phase cells. The New-GFP-induced redistribution of Sup-GFP levels is more pronounced in stationary phase.

demonstrating a causal relationship between accumulation of the larger protein and conversion to [*PSI*$^+$]. This doublet occurs even with naturally occurring full length Sup35p and appears to become more pronounced in stationary phase cells, during which cells are most susceptible to [*PSI*$^+$] induction (Figure 1D)[4]. However, attempts to characterize the precise nature of the difference between the two bands by truncation mapping were unsuccessful, and the failure to observe a clear migration of signal from the large band to the small one in pulse chase experiments made me skeptical of the significance of this finding. Again, I moved on.

Functional characterization of New1p

Another project area in which I have made limited headway concerns the normal biological function of New1p and its role in prion behavior. In the work described in preceding chapters, I transplanted a somewhat arbitrarily delineated chunk of New1p onto the translation termination domain of Sup35p to create an artificial prion-forming protein, New-MEF. A major concern about the physiological significance of this work is whether the full length, chromosomally encoded New1p can form a prion in its own. A close

[4] Note that levels of full-length Sup35p are substantially lower in stationary phase cells than in logarithmically growing cultures.

reading of Chapter 3 will reveal to the careful reader that the [*NU*$^+$] state cannot be maintained in the absence of the New-EF or New-MEF construct. Additionally, [*NU*$^+$] lacks certain characteristics typical of other yeast prions, such as meiotic stability. A firm demonstration that endogenous New1p forms a *bone fide* prion would put paid the quibbles of hostile reviewers.

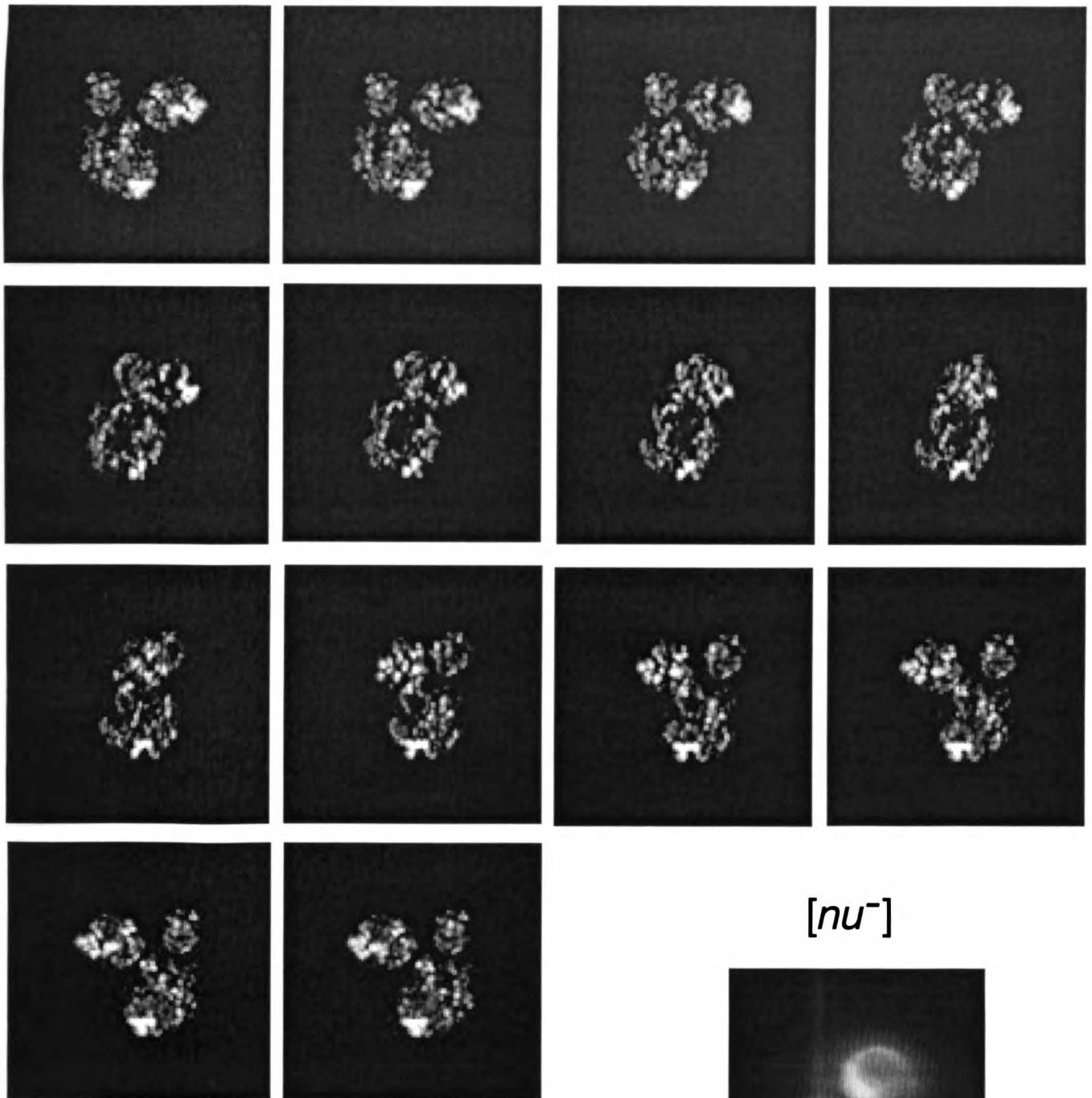
To more directly examine the prion properties of New1p, I compared the behavior of New-MEF and New1p using fluorescent microscopy. I have observed that full length New1p can be incorporated in [*NU*$^+$] aggregates. The chromosomal NEW1 gene was tagged with a C-terminal GFP domain using homologous recombination, and [*nu*$^-$] and [*NU*$^+$] derivates of this strain were examined by wide field fluorescence microscopy (assistance rendered by Orion Weiner). The deconvoluted three-dimensional reconstruction shown in Figure 2 demonstrates that in [*NU*$^+$] strains, full length New1p-GFP forms punctate aggregates distributed throughout the cytoplasm. In contrast, the fluorescent signal in [*nu*$^-$] cells is diffuse. This behavior mirrors that of New-GFP protein expressed at low levels using the SUP35 promoter from a CEN/ARS plasmid. However, if the [*NU*$^+$] strains are allowed to lose the New-MEF plasmid, the full length New1p-GFP reverts to a soluble state; subsequent retransformation with a New-MEF plasmid yields ade- ([*nu*$^-$]) colonies, indicating that the endogenous New1p cannot itself

maintain the [*NU*$^+$] state. Constitutive over-expression of full length New1p from a 2μ plasmid using the strong ADH1 promoter did not improve [*NU*$^+$] maintenance. It thus appears that New1p can participate in prion behavior, but cannot by itself propagate in a prion form. The difference between prion behavior by full length New1p and New-MEF may arise from the presence of inhibitory elements in the YEF2 homology domain of New1p or from an as-yet uncharacterized prion-promoting property of the C-terminal M and EF domains of Sup35p.

My use of the New-MEF chimera arose from need for a selectable phenotype to distinguish between cells with and without prions. Prion forms of Sup35p and Ure2p lead to decreased normal activity of these proteins due to aggregation and inactivation of their functional domains. In order to assess possible prion-associated phenotypes of New1p, it was first necessary to determine what a loss of function of this protein would look like. I disrupted NEW1 in [*psi*$^-$] and [*PSI*$^+$] strains bearing the *ade1-14* nonsense read-through reporter[5] and observed phenotypes consistent with a defect in translation termination. [*psi*$^-$] derivatives were slightly pink in color,

[5] Another useful factoid: one of the first things that I did when I came to the lab was to sequence the *ade1-14* allele. The mutation is W244stop(UGA).

[NU^+] y axis rotation →



[nu^-]

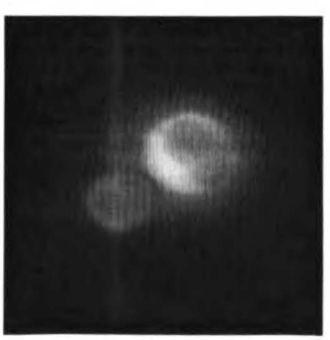


Figure 2. New1p-GFP forms fluorescent foci in [*NU*$^+$] cells.

Deconvoluted three-dimensional reconstruction of [*NU*$^+$] cells expressing New1p tagged with GFP. Each image is rotated ~12.8° around the vertical (Y) axis in order to illustrate the abundant distribution of small fluorescent aggregates throughout the cytoplasm. Compare with [*nu*$^-$] cells shown at bottom right.

while [*PSI*$^+$] isolates were substantially whiter than wild type [*PSI*$^+$] strains (Figure 3). This color change correlated with increased sensitivity to the drug paromomycin, which decreases the efficiency of translation termination and thus exacerbates the effect of mutations that affect this process (data not shown due to poor documentation). I also found that [*NU*$^+$] strains had higher levels of nonsense suppression than [*PSI*$^+$] strains (see Chapter 2); this may arise through inactivation of the endogenous New1p in [*NU*$^+$], compounding the [*PSI*$^+$]-like phenotype with the depletion of another translation factor.

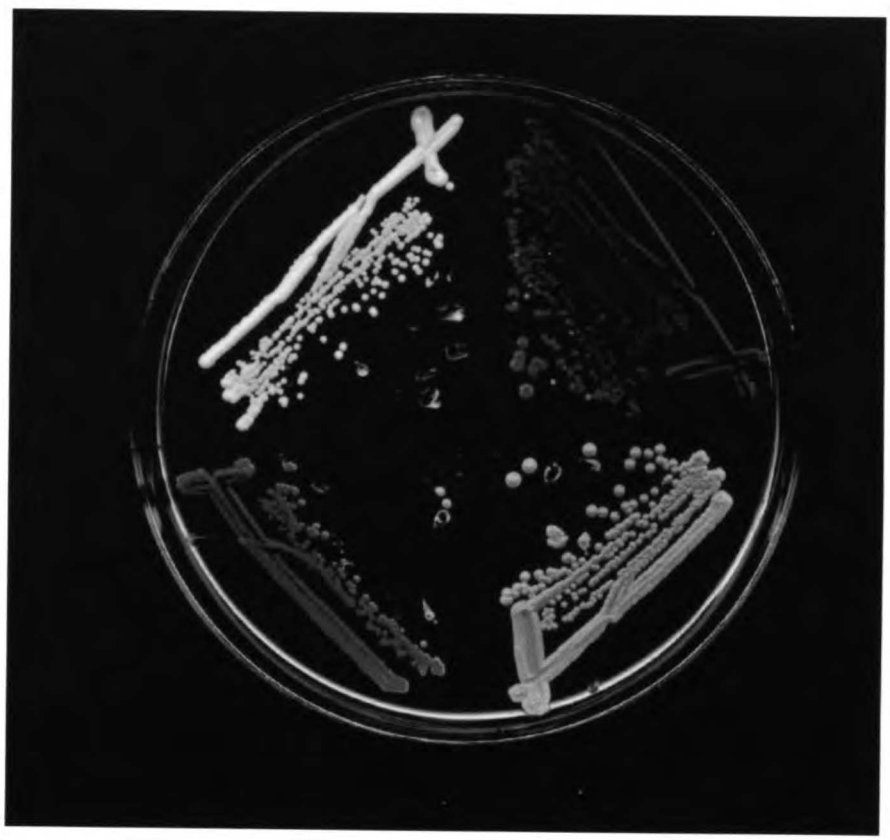
Endogenous New1p may also have a role in the process of [*PSI*$^+$] induction. I observed that [*psi*$^-$] *Δnew1* strains convert to [*PSI*$^+$] at a markedly slower rate when Sup35p is over-expressed. Whereas it takes approximately five days of growth on adenine-free medium for fresh wild type [*PSI*$^+$] convertants to form visible colonies, in a *Δnew1* background, it takes more than ten days to see colonies. The delay in [*PSI*$^+$] induction occurs both in [*psi*$^-$]- [*PIN*$^+$] strains and [*psi*$^-$] [*pin*$^-$] strains with excess New-GFP. This effect is not due to toxicity of Sup35p in *Δnew1* strains, as the growth rate of NEW1 mutant and wild type strains over-expressing Sup-GFP were similar. My best hypothesis about the basis of this delay invokes the action of New1p as a natural competitor substrate for the anti-

[*PSI*$^+$] [PIN$^+$]

Δ*new1*

[*psi*$^-$] [*pin*$^-$]

Δ*new1*



[*psi*$^-$] [*pin*$^-$]

[*PSI*$^+$] [PIN$^+$]

YEPD

Figure 3. Comparison of NEW1 and Δ*new1* phenotypes

Streaks of wild type and Δ*new1* derivatives of [*psi*$^-$] [*pin*$^-$] and [*PSI*$^+$] [*PIN*$^+$] strains are shown. Note the increased pinkness of the Δ*new1* [*psi*$^-$] strain as well as the extreme white color of the Δ*new1* [*PSI*$^+$] strain. These color phenotypes are exacerbated by low doses of paromomycin.

aggregation system that prevents [*PSI*$^+$] induction; the absence of endogenous New1p increases the activity of this factor, making [*PSI*$^+$] induction more difficult. A preliminary comparison of wild type and *Δnew1* extracts from strains undergoing [*PSI*$^+$] induction suggested that Sup-GFP levels are lower in *Δnew1* strains. It also appeared that the delay in [*PSI*$^+$] induction could be modified by deletion of YER049W (PST21), an ORF that I had earlier identified as an enhancer of the [*PSI*$^+$] phenotype. I have failed to follow up on these somewhat interesting observations, but encourage future students to do so.

I have also attempted to recapitulate the prion behavior of the New1p N-terminal domain *in vitro* using purified recombinant protein. I constructed a inducible bacterial plasmid encoding New1p residues 1-153 followed by a six histidine tag and purified this protein using nickel agarose affinity chromatography. New1p$_{1\text{-}153}$-6HIS proved extraordinarily difficult to solublize; even the denaturing 8 M urea lysis buffer used in standard Sup35p prion domain purificates did not liberate the protein from the bacterial pellet. Although I ultimately solublized this protein with 8 M guanidine hydrochloride, dilution into non-denaturing buffer led to its immediate precipitation. I later cloned and purified a version of this protein containing the highly charged M domain of Sup35p between the New1p portion and the HIS tag, and although this protein was

considerably more soluble and easier to purify, I nonetheless observed extensive spontaneous precipitation upon dilution into aqueous buffer. Unlike Sup35p, aggregated New1p did not bind Congo Red dye or Thioflavin-T, and when examined by atomic force microscopy did not form amyloid fibrils, but rather appeared to clump together in an amorphous manner. It is possible that the buffer conditions that I used (those currently in vogue for Sup35p polymerization) are somehow unsuitable for New1p polymerization. However, this finding begs the question of whether it necessary for a protein to form amyloid fibrils in order to be considered a prion.

Is New1p really a prion-forming protein? To a large extent, the New-MEF chimera is an artificial protein with prion properties that are not shared with natural New1p. Furthermore, endogenous New1p has not yet been observed to form a prion in wild type strains; analysis of [*PIN*$^+$] in various yeast strains has invariably linked this property to the prion form of Rnq1p, rather than New1p or other Q/N-rich proteins (my observations and those of Irina Derkatch and Michael Bradley). Nonetheless, New1p has a clear involvement in prion induction and contains a domain that, when transplanted into the appropriate context, exhibits prion behavior. We are not so much interested in proving that New1p is a prion as we are in using it as a tool dissect how prions form and propagate; in this regard, New1p has been extremely useful.

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