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Amyloid Degeneracy in Yeast Prion Strains and Specificity

by

Peter Chien

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

in the

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of the

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by

Peter Chien

dedicated to

BaBa, MaMa, GuGu and TianTian

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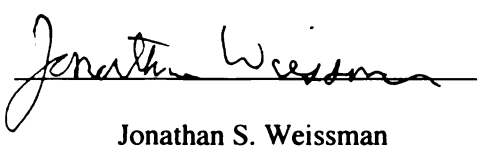
AMYLOID DEGENERACY IN YEAST PRION STRAINS AND SPECIES BARRIERS

Peter Chien

Abstract:

One of the fundamental features of prion diseases is the existence of prion strains in which multiple phenotypes are associated with a single polypeptide sequence. While conventional viral strains are attributed to either host or pathogen genome variation, the protein-only nature of prions requires another mode of encoding. A second hallmark of prion biology is the presence of a species barrier that limits infectivity between even closely related species of prion proteins. This work centers on defining and exploring the origin of the species barrier and the existence of prion strains using the yeast prion [*PSI*$^+$], a self-propagating amyloid aggregate of the translation termination factor Sup35p. [*PSI*$^+$] has proven invaluable as both a genetically tractable and biochemically accessible system for understanding fundamental questions of prion biology. Using this yeast prion system we have shown that *i*) yeast prions are subject to a species barrier, where prion proteins isolated from one species of yeast do not interact with others, *ii*) changes in the conformation of a chimeric prion domain can dictate the species-specificity of the resulting infectious particle, and *iii*) the spectrum of preferred conformations of a prion particle is intimately dependent on sequence and environment. We find that prion strains can arise directly from the ability of a prion protein to adopt multiple amyloid conformations and that these conformations can determine which polypeptide sequences

can add onto the growing aggregate. The existence of these multiple conformations are shown both *in vitro*, through formation of amyloid fibers, and *in vivo*, through the use of transgenic yeast harbouring different species of prion domains. Furthermore, by biasing conformational preference through point mutations or environment, we can generate a *de novo* species barrier between prion domains. Thus, our data suggests that prion strains and species barriers are intimately connected: changes in sequence can influence conformational choice that in turn directly affects its ability to recruit a particular sequence. As amyloid fibers in general can form multiple morphologies and display a measure of sequence specificity, the degeneracy inherent in amyloid formation can explain the origin of prion strains and species barriers.



Jonathan S. Weissman

thesis advisor

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FIGURE LEGENDS

Chapter 1

Introduction.

The introduction is a review submitted to Annual Reviews in Biochemistry, written in 2003 by Angela H. DePace, Jonathan S. Weissman and myself. This review introduces the concept of prion strains and species barriers and covers the evidence for these phenomena in both yeast and mammalian prion systems. Through the discussion of the literature and based in part on our own work, we come to the notion that prion species barriers can be explained as a manifestation of the conformational diversity inherent in amyloid fiber formation. The particular conformation that a protein adopts is intimately dependent on the sequence of the polypeptide; thus, different sequences would naturally adopt different propagating conformations. As amyloid conformations and morphologies are often self-specific (also reviewed here) the prevalence of prion transmission barriers, which occur even between closely related species, becomes a consequence of this facet of protein misfolding. The review concludes with a set of tenets which taken together form a basis for understanding the existence of prion strains and barriers to transmission.

EMERGING PRINCIPLES OF CONFORMATION-BASED PRION INHERITANCE

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ABSTRACT

The prion hypothesis proposes that proteins can act as infectious agents. Originally formulated to explain transmissible spongiform encepatholopathies (TSEs), the prion hypothesis has been extended with the finding that several non-Mendelian traits in fungi are due to heritable changes in protein conformation, which may in some cases be beneficial. While much remains to be learned about the specific role of cellular cofactors, mechanistic parallels between the mammalian and yeast prion phenomena point to universal features of conformation-based infection and inheritance involving propagation of ordered β-sheet rich protein aggregates commonly referred to as amyloid. Here we focus on two such features and discuss recent efforts to explain them in terms of the physical properties of amyloid-like aggregates. The first is prion strains, wherein chemically identical infectious particles cause distinct phenotypes. The second is barriers that often prohibit prion transmission between different species. There is increasing evidence suggesting that both of these can be manifestations of the same phenomenon: the ability of a protein to misfold into multiple self-propagating conformations. Even single mutations can change the spectrum of favored misfolded conformations. In turn, changes in amyloid conformation can shift the specificity of propagation and alter strain phenotypes. This model has important implications for understanding not only prion phenomena but also noninfectious diseases involving toxic misfolded proteins.

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INTRODUCTION

The idea that a protein conformation can replicate itself and therefore serve as a genetic element was first formalized by the prion hypothesis, which seeks to explain an unusual set of neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs). These devastating diseases result in progressive cognitive and motor impairment and are characterized by the accumulation of proteinaceous brain lesions or plaques (1). Sheep scrapie was the first of these diseases to be recognized, but subsequently a set of human diseases, such as kuru and Creutzfeldt-Jacob, were shown to have similar clinical and pathological features. TSEs have now been identified in a wide range of mammals, including cats, cows, mink, deer and elk (2).

Though the TSEs can arise spontaneously or be inherited, they are also infectious (3). The earliest illustrations of infectivity were accidental; sheep scrapie was transmitted to an entire flock during routine vaccination, and kuru was transmitted through ritual cannibalism practised by a tribe in New Guinea. Subsequent experiments showed that human disease could be transmitted to primates and surprisingly indicated that the infectious agent was resistant to classic methods for inactivating nucleic acid. The purification of the infectious agent responsible for scrapie led to the remarkable discovery that it was composed primarily, if not entirely, of protein. Based on this observation, Stanley Prusiner proposed that a novel proteinaceous infectious agent, termed a "prion",

was responsible for these diseases (reviewed in (4)). It was later found that the infectious protein is a ubiquitous endogenous cellular protein, termed PrP, for Prion Protein.

How might an endogenous protein be infectious? In a prescient argument prompted by the need to reconcile the failure to detect nucleic acids in the infectious agent responsible for scrapie with the newly emerging central dogma of molecular biology, J.S. Griffith described three general mechanisms for replication of a protein so that "the occurrence of a protein agent would not necessarily be embarassing" (5). In the first mechanism, a transcriptional activator could be infectious if it were to turn on a normally quiescent gene that participated in a positive feedback loop driving its own production. The second mechanism postulated a change in either protein conformation or multimeric state that cannot occur without a catalyst, such as a preformed multimeric nucleus. The final mechanism invoked an immune response feedback loop. Another mechanism has recently been described by Reed Wickner: a zymogen, or self-activating enzyme can been infectious if an active form is introduced into a pool of otherwise stably inactive protein (6).

One of above mechanisms, propagation of conformational change, appears to underlie the mammalian TSEs. During purification of the infectious scrapie agent, a β-sheet rich insoluble protease-resistant fragment of PrP was associated with highly infectious preparations. Surprisingly this form is covalently identical to the normal cellular form of PrP, but in uninfected animals PrP is alpha-helical, soluble and protease sensitive (Figure 1). A variety of observations now support a model where the scrapie-

associated form of PrP, termed PrP^{SC}, is transmitted by conformational conversion of the normal cellular form, called PrP^{C}. Though two Nobel prizes have been awarded for research on TSEs, the biology of mammalian prion diseases is still hotly contested (7). Specifically, the size and nature of the infectious particle remain unresolved due to experimental limitations. First, the specific activity of purified material is extremely low (8). Second, infectious preparations without protease-resistant PrP^{SC} have been found (9). Third, and most challenging, recombinant infectious material has not yet been produced *in vitro* to provide the formal proof of the protein-only hypothesis. Together, these technical limitations have left lingering questions whether other components, such as chaperones, small molecules or even RNAs (10) could play a role in prion infection.

Despite these questions about the mechanisms of mammalian TSEs, it has become clear that proteins can serve as genetic elements and that prions are more widespread in biology than previously thought. In 1994, Reed Wickner proposed that the behavior of two non-Mendelian cytoplasmically inherited traits in *S. cerevisiae*, [*PSI*$^{+}$] and [*URE3*], could be explained by a prion-like mechanism where an alternate protein form does not cause disease but does "infect" daughter cells as they bud from the mother (11). This model was based on three remarkable features shared by [*URE3*] and [*PSI*$^{+}$]. One, propagation of [*URE3*] and [*PSI*$^{+}$] are dependent on the continuous expression of an associated gene, *URE2* and *SUP35* respectively, yet their phenotypes mimic loss-of-function mutations in these genes. Two, [*URE3*] and [*PSI*$^{+}$] can be cured by growth on guanidine hydrochloride and can return to the prion state without any changes in the genome. Three, overexpression of Ure2p and Sup35p increases the frequency of *de novo*

[*URE3*] and [*PSI*$^+$] appearance. Wickner's model elegantly explained these observations by postulating that overexpression results in a novel prion form of the protein. The prion form is self-propagating which allows inheritance, and inactivating which results in the apparent loss-of-function phenotype. This model has been confirmed and expanded by the work of multiple labs and it is now established that both [*PSI*$^+$] and [*URE3*] are due to the self-propagating aggregation of Sup35p and Ure2p, respectively (12, 13). More fungal prion domains have been discovered subsequently, including [*RNQ*$^+$] – also known as [*PIN*$^+$], [*NU*$^+$], and [*Het-s*] – these have been comprehensively reviewed elsewhere (14, 15).

The yeast prions have provided genetically and biochemically tractable systems for studying prion behavior, greatly facilitating studies on the mechanism of conformation-based inheritance and infection (12, 13). [*URE3*] and [*PSI*$^+$] are the best characterized and both offer accessible *in vivo* and *in vitro* experimental systems. *In vivo*, genetic screens can exploit the nitrogen uptake phenotype of [*URE3*] yeast or the nonsense suppression phenotype of [*PSI*$^+$] yeast (Figure 2). *In vitro*, propagation of [*URE3*] and [*PSI*$^+$] are modeled by the formation of amyloid fibers. Both Ure2p and Sup35p are modular proteins, with their prion activity localized to an amino-terminal glutamine/asparagine rich domain separable from domains responsible for their normal cellular function (Figure 2). These purified prion domains spontaneously form amyloid fibers only after a characteristic lag-phase that can be eliminated by the addition of preformed seeds, mimicking propagation *in vivo*. Importantly, formal proof of the prion hypothesis has come from studies with [*PSI*$^+$] and [Het-s]. When introduced into cells,

amyloid seeds generated *in vitro* from purified recombinant Sup35p or Het-s* are able to cause *de novo* formation of the [*PSI*$^+$] and [Het-s] states respectively (16, 17).

While there are critical differences in the cellular location and phenotypic consequences between mammalian and yeast prions, they share a remarkable number of common mechanistic features. In this review, we will compare and contrast these systems in an effort to build a general model for conformation-based infection and inheritance. We will first consider that both mammalian and yeast prions appear to be due to the propagation of β-sheet rich aggregates that resemble amyloid fibers. In contrast to disordered amorphous aggregates, amyloids are highly ordered fibrillar structures, formed by a wide variety of polypeptides with no homology in either their native structures or in their amino acid sequence (Figure 3). In many cases these amyloids are self-propagating; however, in general, amyloids are not infectious. Thus, an unresolved question is what distinguishes prions from this larger class of misfolded proteins. We will next consider that both mammalian and yeast prions display multiple strains, in which infectious particles composed of the same protein give rise to distinct phenotypes. This strain phenomenon has been difficult to reconcile with the protein-only hypothesis, but evidence is accumulating from both the prion and amyloid fields that a single polypeptide can form multiple distinct conformations that may provide the structural basis for strain diversity. We will then examine the sequence specificity of prion propagation, which manifests in both the mammalian and yeast systems as a "species barrier" inhibiting transmission between even highly related species. Finally, we will review the evidence that strains and species barriers can result from the same

underlying process, namely that a single polypeptide can form multiple self-propagating states. These different conformations can lead to distinct strain phenotypes and can determine the sequence specificity of prion propagation.

AMYLOID-LIKE SELF-PROPAGATING PROTEIN AGGREGATES UNDERLIE PRION INHERITANCE

The fundamental requirement of the prion hypothesis is that a protein be capable of adopting a state that can initiate and sustain its own replication. While the cellular machinery for transcription and translation are used to generate new polypeptides, the infectious protein must contain enough information to direct production of the prion rather than the normal cellular form. Abundant evidence has accumulated that both mammalian and yeast prions accomplish this by directing conformational change of a normal cellular host protein into an alternate prion conformation. These alternate conformations are β-sheet rich multimers and resemble a broader class of ordered protein aggregates termed amyloids. Amyloids are associated with a variety of non-infectious neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, as well as range of systemic amyloidoses (18). Here we review the evidence that prions operate by directing conformational change of a host protein and what is known about the formation and structure of these alternate conformations. Finally, we will explore the steps of prion replication to explain why prions are an infectious subset of the larger class of proteins that misfold into amyloid. Specifically, we will focus on the infectivity requirements beyond simple self-propagating protein structures, a feature shared by many amyloids.

Evidence for conformational changes in mammalian prions

The first evidence indicating that conformational change was involved in prion diseases arose during the purification of the infectious scrapie agent. A protease resistant protein fragment was found to copurify along with infectivity (8, 19). Subsequent cloning of this fragment revealed that it was part of a larger 33-35kDa host glycoprotein, encoded by the *PRNP* gene (20-22). The normal cellular version of this protein, called PrP^{C} for cellular PrP, is distributed throughout many visceral tissues, is soluble and highly sensitive to proteinase K digestion. However, in infected animals an insoluble form of PrP is also present, called PrP^{SC} for scrapie PrP. PrP^{SC} accumulates in aggregates and plaques in the brain and digestion with proteinase K cleaves only its first 66 amino-terminal residues, leaving a fragment referred to as $PrP^{SC27\text{-}30}$ with an SDS-PAGE mobility of 27-30 kDa.

The difference between PrP^{C} and PrP^{SC} appears to reside completely in their conformations. Though mutations in the nucleic acid genome can increase rates of spontaneous disease (2), the infectious disease occurs in the absence of such mutations. Moreover, systematic analysis of post-translational modifications have failed to find any evidence that covalent modifications underlie formation of the infectious form (23, 24). By contrast, extensive evidence argues that PrP^{C} and PrP^{SC} adopt distinct conformations. For example, in addition to the protease resistance and solubility mentioned above, the two conformers vary in the exposure of a number of different epitopes (25) and have

dramatically different thermodynamic stabilities (26) and secondary structure content. The structures of human, hamster, bovine and mouse PrP^C have been solved by NMR (27-30), and all are highly similar, predominantly alpha-helical folds. PrP^{SC} on the other hand is predominantly β-sheet, as revealed by FTIR studies (31).

Extensive evidence reviewed elsewhere implicates the conversion of PrP^C to PrP^{SC} in disease progression (32). *In vivo*, $PrP^{0/0}$ mice are not susceptible to prion infection, arguing that conversion of the endogenous protein is required to develop disease (33, 34). Furthermore, the lag time before developing disease is dependent on the concentration of PrP in the host (34-37). The infectious process can be recapitulated in cell culture using a neuroblastoma N2a cell line (38). *In vitro*, extracts enriched in PrP^{SC} can convert recombinant PrP^C to a protease resistant form called PrP^{RES}, and this material exhibits similar specificity to that seen *in vivo* (39, 40). It has also been reported that shearing aggregates during the polymerization reaction increases the yield of protease resistant material (41, 42). Nonetheless, to date *de novo* infectious material has failed to be created *in vitro*. A second caveat is that infectious prion diseases have been observed in the absence of detectable protease-resistant PrP^{SC} aggregates (9, 43, 44). However, the question remains whether this absence is due to a titer of aggregates below detection limits, to a formation of an infectious conformation that is genuinely protease-sensitive, or to some more radical departure from the idea that conformational changes are necessary for generating infectivity.

Evidence for conformational changes in fungal prions

Yeast prions, like mammalian prions, are characterized by the presence of an alternate conformation of a normal cellular protein. All fungal prion proteins yet identified have been shown using either differential sedimentation or size-exclusion chromatography to form high molecular weight complexes specifically in prion-containing cells (45-53). However, the degree of aggregation *in vivo* can vary with genetic background (54, 55) or the expression level of the cognate prion protein (56). Aggregated protein can be visualized in intact cells by generating prion-GFP fusion proteins that are soluble and distributed evenly throughout the cytoplasm in wildtype cells, but are organized into punctate foci stainable by the amyloid-specific dye thioflavin-S, in prion-containing cells (46, 49, 50, 57-59). Ure2p has also been visualized by thin-section EM followed by immunogold staining and shown to form short cytoplasmic fibrils specifically in [*URE3*] yeast (60). In most cases, these aggregates have been shown to be highly stable and to have altered resistance to protease digestion (45-47, 51). Finally, *de novo* formation of these aggregates is slow, but once formed they are stably inherited by daughter cells during mitosis.

The fungal prions have proven to be far more amenable to reconstitution *in vitro* than the mammalian prion system. Extracts from [*PSI*$^+$] yeast can catalyze conversion of soluble Sup35p, while extracts from [*psi*$^-$] yeast do not have this activity (61). Moreover, for Ure2p, Sup35p and HET-s, inheritance can be modeled using purified protein. Following a characteristic lag phase, these proteins spontaneously form amyloid-like

aggregates. Importantly, the lag phase can be eliminated by the addition of small amounts of preformed fiber seed (62-64). A number of lines of experiments argue that this seeding effect underlies prion inheritance *in vivo*. For example, mutations in Sup35 which affect aggregation *in vivo* have parallel effects on the *in vitro* reaction (46, 65, 66). More directly for Sup35p and HET-s, it has been possible to create aggregates *in vitro* from recombinant protein and use these to convert wildtype cells to the prion state (16, 17). These experiments have provided the most complete evidence to date for the protein-only hypothesis.

Beyond supporting the prion hypothesis, this facile *in vitro* system allows more detailed mechanistic studies of prion conversion. Three questions stand out. One, what is the aggregation state of the infectious material? Specifically, are fibers necessary for infection or are they merely an assembly byproduct of conformational conversion? What is the minimum size of an infectious particle? Two, when does conformational conversion occur? Monomers or oligomers could undergo spontaneous conformational conversions in solution that are subsequently stabilized by assembly into polymers, or conformational conversion could be driven by the assembly process itself. Three, what is the rate-limiting step in prion formation? Nucleated polymerization models argue that the formation of a multimeric nucleus is the slow step, while templated assembly models argue that conformational conversion is rate-limiting, though these two are not necessarily mutually exclusive. Detailed coverage of the literature addressing the conversion reaction is beyond the scope of this review, but we encourage interested

readers to consult recent reviews (67-69), and research papers addressing the subject (70-74).

Prion aggregates resemble amyloid fibers

Recently it has become clear that a wide range of unrelated proteins form structurally similar β-sheet rich aggregates, often referred to as amyloids. Amyloids have received an enormous amount of attention due to their association with a wide variety of protein misfolding disorders, including neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's (75). They are also a number of systemic amyloidoses, where fibers accumulate in the periphery due to the aggregation of such proteins as lysozyme, transthyretin (TTR), immunoglobulin light chain, β2-microglobulin and islet amyloid protein, or amylin (76). Furthermore, a number of non-disease associated proteins, such as acylphosphatase and the SH3 domain from PIP_3 kinase, have been shown to form amyloid under mildly denaturing conditions (77, 78). The ability of such diverse polypeptides to form amyloid argues that this fold is generally accessible to polymers of amino-acids, perhaps because it is stabilized by main-chain rather than side-chain interactions (18).

Despite the variety in amyloid-prone proteins and their aggregated states, amyloids share similarities that make it useful to discuss them as a family of related structures (Figure 3). Amyloid fibers are characterized by a set of fiber diffraction reflections indicating that the β-sheets are organized in a cross-β fold where the strands

of the sheets run perpendicular to the fiber axis while the sheets run parallel to it (79-81). The repeating β-sheet structure allows the binding of the hydrophobic dyes thioflavin-T and Congo Red, both of which are commonly used to monitor amyloid formation *in vitro*. Amyloids are often composed of multiple thin protofilaments that can associate in a variety of ways to create mature fibers with a range of diameters and helical twists (reviewed in (82)). Currently, cryo-electron microscopy has yielded the most detailed structural model of an amyloid and indicates that protofilaments can be arranged around a hollow core (83) (Figure 4). Multiple folds can satisfy the constraints of a cross β-sheet structure (81) such as the β-helix shown in Figure 4. Most generally, amyloids are uncapped β-sheets that can incorporate new protein on their edges, leading to a fiber of defined diameter but unlimited length. In fact, well behaved β-sheet rich proteins appear to avoid aggregation by protecting their β-sheet edges with a variety of strategies (84).

Though the heterogeneity and insolubility of prion aggregates have made high-resolution structural studies difficult, they are known to share many features with amyloid (62-64, 85). Recently, reconstruction from electron micrographs of 2D crystals of PrP^{SC} present in infectious preparations has provided enough constraints to propose structural models (86). Additionally, a crystal structure of a PrP dimer has been solved, and suggests how subunits might assemble into a fiber (87). Sup35p fibers give rise to the stereotypic amyloid cross-β diffraction pattern when subjected to fiber diffraction (71), and Ure2p fibers give rise to this pattern after being subjected to heat, though Ure2p may assemble into native-like filaments under physiological conditions (88). Both Sup35p and Ure2p fibers appear to consist of a central core made up of the prion domain,

with globular domains corresponding to the remainder of the protein decorating the periphery (62, 89) (Figure 3). Taken together with the self-propagating behavior of amyloid, the structural similarities between amyloid and prion aggregates suggest that propagation of β-sheet rich amyloid-like core could provide the molecular mechanism responsible for prion growth.

Self-propagating aggregates are not sufficient for infection/inheritance

Amyloid fibers formed by many proteins are self-seeding (90-94) but few are infectious. For example, Aβ, the peptide whose aggregation is intimately correlated with Alzheimer's disease (95), exhibits stereotypical self-propagating behavior *in vitro* forming amyloid after a lag phase that can be eliminated by the addition of preformed fibers (67). Yet, Alzheimer's disease is not transmissible to primates or rodents (96, 97). What then is unique among the prion-associated amyloids that allows them to be infectious? We consider the steps of aggregation and transmission in Figure 5, comparing PrP and [*PSI*$^+$].

Initially, a self-propagating aggregate must form spontaneously. This is a step common to all of the amyloid diseases; in fact, most cases of Alzheimer's and Creutzfeldt-Jacob occur spontaneously in patients without any genetic predisposition to the disease (3). Yeast prions occur spontaneously only rarely but are stable once formed (14). In mammals, mutations can accelerate the rate of spontaneous aggregation, as can overexpression of the aggregation-prone protein (34-37). In the case of yeast, truncations

and expansions can accelerate the rate of spontaneous occurrence (66), and overexpression greatly increases the rate of prion formation (98). Finally, exposure to environmental factors such as metals and pesticides may also facilitate protein aggregation (99, 100).

Next, the newly-formed prion must replicate itself. This involves two separable steps: growth of the infectious particle by addition to the aggregate and amplification of the number of infectious particles. Growth of the infectious particle comes about through recruitment and assembly of new protein onto the prion. However, this process alone would only lead to an increase in mass of protein in the prion form without net increase in the number of catalytic surfaces. Therefore, new infectious particles must somehow be released from the aggregate, either by spontaneous shedding or division by a cellular factor. Though how this is accomplished by mammalian prions is unclear, [*PSI*$^+$] aggregates have been postulated to be divided by the chaperone Hsp104p. The primary evidence for this model is the peculiar relationship between [*PSI*$^+$] and Hsp104p where both deletion and overexpression of *HSP104* interferes with [*PSI*$^+$] propagation (101). Hsp104p is normally involved in the rescue of aggregated protein with the help of Hsp70p and Hsp40p (102), and indeed may play a general role in prion propagation, as deletion of *HSP104* cures all known yeast prions (14, 15). Division of aggregates could be another step differentiating transmissible and non-transmissible aggregates; if aggregates are too stable to either release small units or to be degraded by chaperones, they would never exponentially amplify during infection or inheritance.

Finally, these aggregates must be transmitted into a naïve host and reach a pool of substrate protein. Mammalian prions can be ingested orally, as evidenced by the Mad Cow epidemic and the transmission of kuru through ritualistic cannibalism. The prion infection then reaches the central nervous system (CNS) apparently through the lymphoid tissue. Once in the CNS, prions are able to spread from one cell to another presumably due to the presence of PrP^C is exposed on the surface of the cell (103). Yeast prions are transmitted naturally during cell division or experimentally using cytoplasmic mixing. This is clearly a critical step in differentiating between infectious and non-transmissible amyloids (104). Protein aggregates could vary in their ability to circumvent the body's defenses by being differentially susceptible to degradation and/or transport. There is recent evidence that systemic amyloids can also be transmissible when administered either orally or intraveneously after an inflammatory stimulus, arguing that under the right conditions, more aggregates may be proven to be infectious (105).

In addition to this growth and replication cycle prion aggegates also cause a phenotypic change in their hosts. The need to distinguish phenotypic output from prion replication is emphasized by recent work which shows that high titers of prions can exist without development of clinical symptoms (106). In the case of amyloid-related neurodegenerative diseases, the mechanism of toxicity and their tissue specificity must also be accounted for (107). In systemic amyloidoses, disease may be caused by mechanical disruption due to enormous amyloid burden, as simple removal of amyloid deposits alleviates symptoms (108). In some cases it may be that amyloid fibers are not toxic, but instead are an inert repository for improperly folded proteins, and that the

intermediates along the pathway to amyloid formation are neurotoxic (107). Indeed, partially unstructured oligomers of both Aβ and α-synuclein are toxic to cells (109, 110), as are partially unstructured oligomers of an SH3 domain which is not associated with any known disease (111). For [*URE3*] and [*PSI*$^+$], the relationship to phenotype is more straightforward; sequestration of the prion protein in aggregates leads to a phenotype similar to a loss-of-function (11, 112). In fact, the prion domain from Sup35p can be fused to other proteins to create novel prion elements with phenotypes due to inactivation of the fusion protein (49, 66). However, a simple inactivation model is not sufficient to explain all fungal prions as [Het-s] and [*PIN*$^+$] lead to a gain-of-function phenotypes (51, 113, 114).

PRION STRAIN VARIATION

One of the most fascinating and perplexing features of prion biology is the existence of multiple prion strains, wherein infectious particles composed of the same protein give rise to a range of prion states that vary in incubation time, pathology and other phenotypic aspects. Observation of strain variability preceded the prion hypothesis, and in fact was originally used as evidence for the existence of a nucleic acid genome in the infectious particle. Strain variation was postulated to be due to mutations in this genome. In the context of the proposal that transmissible encephalopathies are due to propagating conformational changes in a prion protein, one must postulate that a single polypeptide can misfold into multiple infectious conformations, at least one for each

phenotype. As disconcerting as this idea may be, there is increasing evidence from studies of both fungal and mammalian prions that it is indeed true. Nonetheless, there remain many unresolved questions regarding the origin of prion strains and their relationship to phenotype. For instance, what role do cellular factors play (10, 115)? Do prion conformational differences lead to strain variation or simply reflect some other mechanism that actually encodes strain diversity? Formal proof of the conformational basis for prion strains has not yet been established. These questions remain largely because it has not yet been possible to fold a purified polypeptide into distinct conformations, introduce these into a naïve organism, and demonstrate that they lead to distinguishable phenotypes.

Strain variability in mammalian prions

Strain variability has always been closely associated with transmissible spongiform encephalopathies. Classic experiments in transmission of sheep scrapie to goats led researchers to group isolates according to clinical syndromes, such as 'drowsy' and 'scratchy' strains (116). Material derived from these animals could infect mice where these strains would propagate with distinct clinical and pathological parameters such as patterns of brain lesions (117) and lag in incubation times. Use of isogenic mouse models made it unlikely that this variation arose from host genome polymorphisms (118).

With the identification of the PrP protein as the core component of the infectious particle, classification of strains could focus on molecular analysis of differences in the prion protein. Differences in secondary structure content (119), thermal stability (26, 120) and epitope exposure (121) of PrP^{sc} isolates can be used to distinguish prion strains. Post-translational modifications, such as glycosylation and attachment of GPI anchors, also show differences among known prion strains (2, 122, 123). Whether these covalent modifications modulate prion strains or reflect an inherent diversity among strains is still unknown.

Conformational differences distinguish mammalian prion strains

In light of the hypothesis that prions result from propagation of an infectious conformation, much of the effort in analyzing strains has focused on identifying strain-specific conformational differences in the prion protein. Initial evidence for such differences came from strains of transmissible mink encepholopathy (TME). PrP^{SC} accumulated within the brains of infected minks and showed distinct proteolysis patterns and glycosylation profiles that correlated with different strain types. Upon injection of this material into näive hosts, not only did the newly infected animals exhibit strain-specific brain lesions and incubation times, but the converted PrP^{SC} retained the proteolytic digestion pattern of the inoculum (124). Similarly, transmission of human-derived infectious material into transgenic mice expressing a human-mouse chimera produces PrP^{SC} with hallmarks of the original strain, including protease-sensitivity and glycosylation patterns (125).

A series of cell-free experiments have provided evidence that these protein conformations are sufficient to mediate their own propagation. Caughey and coworkers developed an *in vitro* system where brain-derived PrP^{SC} mixed with PrP^{C} converts PrP^{C} to a protease-resistant PrP^{SC}-like state, called PrP^{RES} (39, 42). Paralleling the *in vivo* experiments, TME prion strains convert PrP^{C} to a PrP^{SC} similar to the initial strain, as defined by proteolysis and extent of glycosylation (126). While other cellular factors such as chaperones or a potential Protein X (127, 128) may be required for robust propagation of strain differences *in vivo*, the above observations suggest that the particular prion conformation can mediate strain-specific conversion of PrP^{C}.

Strain variability in yeast prions

The existence of strains appears to be a ubiquitous feature of prions, independent of the specific prion protein, the types of posttranslational modifications, or the cellular site of conversion. Strain variability in fungal prions affects a range of different properties including the strength of the associated phenotype, mitotic stability and the dependence on molecular chaperones. Fungal prion strains were discovered during analysis of *de novo* induction of [*PSI*$^{+}$] by overexpression of Sup35p. Remarkably, inductants showed clear and heritable differences in color phenotype, caused by differences in the strength of nonsense suppression (98, 113). Genomic mutations cannot account for these differences; once a particular [*PSI*$^{+}$] variant was cured, the full spectrum of strains was reproduced upon reinduction. This variation among prion states

has also been documented in Sup35p derived from other species. For instance, [*PSI*$^+$] elements arising in *S.cerevisiae* expressing the *Pichia methanolica SUP35* showed phenotypic variation, and can be distinguished by their differential sensitivity to a host of chaperones, such as Hsp70p's and Hsp40p's (53, 129, 130). Chaperone discrimination is also seen with a chimeric prion domain derived from *C. albicans* and *S. cerevisiae* Sup35p; overexpression of Hsp104p results in differential curing of these prion variants (P. Chien and J. Weissman, unpublished).

Although [*PSI*$^+$] prion variants are the best characterized, similar variants have been seen in all yeast prions examined so far. *De novo* induction of the [*URE3*] prion results in variants distinguished by the strength of the associated phenotype, and their susceptibility to curing by expression of an inhibitory fragment of Ure2p (48). The [*PIN*$^+$] element, mediated by self-propagating aggregates of the Rnq1p protein, also shows phenotypic variation. Unlike [*PSI*$^+$] and [*URE3*], the [*PIN*$^+$] phenotype is gain-of-function; [*PIN*$^+$] is required for efficient induction of [*PSI*$^+$] by overexpression of Sup35p (15, 49, 131, 132) and deletion of *RNQ1* does not mimic this phenotype. 'Strong' [*PIN*$^+$] elements can generate high numbers of [*PSI*$^+$] cells upon, while 'weak' variants are not as efficient at conversion (114).

Yeast prions strains modulate solubility of the prion protein

An important link between yeast prion strain phenotypes and the conformation of the prion protein came from studies of variant-specific differences in the solubility of the

endogenous prion protein (113, 133, 134). This was first shown using [*ETA*], a non-mendelian genetic element isolated through synthetic lethality with particular alleles of translation release factors (135). Elegant experiments by Zhou et. al. (133) showed that [*ETA*] was a weak variant of [*PSI*$^+$] and distinguished primarily by a reduced level of Sup35p aggregation relative to strong [*PSI*$^+$] strains (133). Other [*PSI*$^+$] variants have now also been characterized and been shown to have similar differences in the degree of aggregation of Sup35p. Importantly, these variants propagate faithfully, and are largely independent of the yeast genetic background (134). The variants of [*PIN*$^+$], which show differential ability to promote [*PSI*$^+$], also show differences in the amount of aggregated Rnq1p but there is no clear correlation between that phenotype and the degree of aggregation (114). Therefore changes in the relative fraction of aggregated protein can result in prion variants but it is not the only possible mechanism for phenotypic diversity.

In vitro analysis of yeast prion strains

Further evidence that [*PSI*$^+$] variants are encoded by different prion conformations came from two lines of experiments. The first took advantage of an extract-based system where [*PSI*$^+$] extracts containing aggregated Sup35p were mixed with [*psi*$^-$] extracts containing only soluble Sup35p that was converted to an insoluble form after incubation (61). Conformational differences between variants could be propagated in a cell-free system. When extracts from [*PSI*$^+$] variants showing differential sedimentation profiles of Sup35p were used to 'seed' [*psi*$^-$] extracts, the newly aggregated material showed the same sedimentation as the original variants (134).

Further experiments validate this notion as Sup35p aggregate-containing extracts generated from either strong or weak [*PSI*$^+$] variants showed different seeding efficiencies in *in vitro* polymerization reactions. However, this difference was lost when the newly polymerized material was used as seeds for secondary rounds of *in vitro* reactions, raising the possibility that faithful propagation of different Sup35p conformation *in vivo* depends on host factors (136).

Yeast prion proteins adopt multiple self-propagating forms

Work with pure protein has established that both Ure2p and Sup35p are able to adopt multiple self-propagating conformations. Spontaneous polymerization of either Sup35NM (the amino-terminal domain of Sup35p) (62) or Ure2p protein produces a range of amyloid fiber types (137). Even though the specific conformational differences between fiber types have not yet been determined, characteristics correlated with strain phenotypes such as kinetics and seeding specificity have been measured for Sup35NM fibers. When an atomic force microscopy (AFM) based assay was used to measure growth from individual Sup35NM fibers, it was found that the purified polypeptide spontaneously forms multiple kinetically distinguishable fiber types. These could be sorted into a discrete number of classes on the basis of their growth polarity and elongation rate (Figure 6). Both the number of distinguishable fiber types and their relationship to protein aggregation rates suggest that these differences are well-suited to account for [*PSI*$^+$] strain variation *in vivo* (138). Other *in vitro* work with a chimeric Sup35p system demonstrates that a single protein can form multiple biochemically

distinguishable conformations with properties that reflect their *in vivo* strain phenotypes (Figure 7) (139, 140).

Structural polymorphism is a common feature of amyloid aggregates

While the notion that prion strains are due to multiple infectious conformations was radical when first proposed, it is now clear that many proteins misfold into a variety of aggregates. This is especially true of amyloids, where even in the same polymerization reaction a single polypeptide can adopt multiple fiber types, distinguishable by their ultrastructural properties such as number of protofilaments and helical pitch as shown in Figure 6 (141-145). Just as crystal growth is intimately dependent on the nature of the solution, changing reaction conditions can shift the relative populations of morphologically distinct fibers. For example, amyloid forms of an SH3 domain were shown to be highly sensitive to pH (83, 146). Aggregation of an Alzheimer's Aβ-derived peptide also showed strong dependence on pH, forming thicker ribbon-like fibers at higher pH (147). Changes in temperature can also alter the range of fiber morphologies as illustrated by work with polyglutamine peptide aggregates (74) and yeast prion amyloid fibers (140).

In addition to reaction condition, covalent changes in the polypeptide, such as mutations or chemical modifications, can also modulate the spectrum of misfolded protein conformations. Mutations in Aβ affect the ultrastructural packing of amyloid protofilaments and overall length of fibers when compared to wildtype (141, 148-150).

Mutations in light chain domains also alter the morphology of amyloid fibers (151). Formation of non-fibrillar intermediates along the pathway to amyloid formation can also be influenced by changes in primary structure. For example, mutations in α-synuclein correlating with early onset of Parkinson's disease have been linked to accelerated formation of toxic protofilament structures that are normally not present in wildtype polymerization reactions (152-154). Finally, the propagating form of yeast prion proteins can also be influenced by mutations (140), resulting in a shift in aggregate stability and in species-specific seeding processes (see later chapters for discussion).

How do even relatively small changes in environment or in primary structure cause shifts in final aggregate morphology? Models for amyloid structure may shed light on the origin of this effect. The core of the amyloid structure is thought to consist of multiple layers of closely packed β-sheets. Morphological variants could arise from differences in sidechain packing, register or topology of β-sheets, or quartenary structures. Though amyloid fibers are notoriously difficult to study using classical methods such as x-ray crystallography or NMR, high resolution structural studies are clearly needed.

SEQUENCE DEPENDENT PRION TRANSMISSION: THE 'SPECIES BARRIER'

Passage of transmissible spongiform encephalopathies between species has long been known to be limited by 'species barriers' (155) and analogous barriers to

propagation exist in the yeast prion systems (52, 53, 129, 156, 157). The primary structure of the prion protein is a critical determinant of the specificity of propagation, as the inhibition of cross-species infectivity is intimately dependent on the degree of similarity between the sequences of the two prion proteins (2, 158, 159). Indeed, even point mutations or allelic variants can have dramatic effects on the specificity of prion propagation (160-164). While host factors may play an important role, increasing evidence from *in vitro* studies argues that the growth of amyloid-like aggregates can account for much of the observed specificity. In general, prion infectivity is also highly dependent on the prion strain in question; we will address this feature in the following section, while focusing here on primary structure differences.

Mammalian species barriers *in vivo*

Species barriers are common among the TSEs. Sheep scrapie isolates are delayed in transmission to goats (155), and human TSEs do not easily infect laboratory mice (165). Systematic exploration of this phenomenon has been greatly facilitated by the establishment of scrapie in transgenic mice, where a species barrier greatly slows prion transmission between Syrian hamsters and mice. From these studies, the sequence of the PrP protein has emerged as a critical determinant of cross-species transmission (158, 159). When PrP^{SC} isolated from Syrian hamsters was intracerebrally injected into hamster hosts, the animals rapidly came down with disease, while mouse hosts showed no clinical symptoms after inoculation with the same material. A transgenic mouse expressing a copy of the Syrian hamster *PRNP* gene in addition to the endogenous copy

was now highly susceptible to both hamster and mouse innoculum (158). Strikingly, inoculation with mouse-derived prions resulted in formation of exclusively mouse prions and hamster prions resulted in exclusive formation of hamster prions (159). However, interpretation of these results is complicated somewhat by the recent finding that high prion titers can exist in the absence of clinical disease features (106).

Since these classic studies, several transgenic experiments have confirmed the intimate relationship between the sequence of the prion protein and specificity of transmission (2, 166, 167). Nonetheless, other studies establish that in some contexts it is not the sole determinant. For example, transgenic mice expressing a human copy of *PRNP* in addition to their endogenous mouse copy (Tg(Hu) mice) are immune to human prions (127). Ablation of the mouse *PRNP* gene in Tg(Hu) mice makes them susceptible to human prions, whereas mice expressing a mouse-human chimera PrP (Tg(MH2M) mice) are susceptible to human prions independent of the presence of the endogenous mouse copy (128). These data led to the suggestion that a species-specific factor (known as protein X) is necessary for prion susceptibility. In Tg(Hu) mice this factor would bind selectively to the wildtype mouse PrP^C protein, preventing proper conversion of the human PrP^C. On the other hand, Tg(MH2M) mice were postulated to have both the human derived sequence necessary for conversion and the recognition epitopes required for binding the prion-promoting factor (128).

Role of polymorphisms in prion transmission

Even within a single species, allelic variants of PrP affect mammalian prion transmission. The effects of genetic background on scrapie susceptibility were observed as early as 1959 by W.S. Gordon who found that some breeds of sheep were particularly sensitive to scrapie. In humans, familial forms of prion diseases are often associated with particular alleles of *PRNP* (2). Although mutations can result in general acceleration of prion onset and transmission, there is also a potential role for *PRNP* alleles to modulate prion transmission specificity. These observations have now been more extensively studied using transgenic mice (161, 168), cell culture (169), and cell-free extract systems (164) where it has been shown that single substitutions in primary structure can determine susceptibility and specificity to prion infection.

Sequence-specific mammalian prion replication *in vitro*

Conversion experiments in cell-free extract systems have helped define the molecular nature of species specificity in prion transmission (40, 42, 170). Incubation of mouse or hamster PrP^{SC} extracts with recombinant PrP^{C} protein from the same species resulted in conversion of the PrP^{C} to a protease resistant form remiscent of PrP^{SC}. However, hamster PrP^{SC} could not convert mouse PrP^{C} suggesting that the specificity of prion propagation resulted from the ability of the infectious particle to bind to and convert soluble PrP^{C}(40). A similar *in vitro* result was also described for species-specific transmission of chronic wasting disease from cervids to other mammals (170). While other factors necessary for prion replication (127, 128) may be present in these extracts,

it seems likely that sequence specific and direct interactions between PrP^{SC} and PrP^{C} underlies much of the prion species barrier.

Transmission of [*PSI*$^+$] is highly sequence specific

Barriers inhibiting yeast prion transmission have been extensively studied using the yeast prion [*PSI*$^+$]. Cloning of *SUP35* genes from a broad range of budding yeast revealed that although the exact sequence of the amino-terminal domain varies, the features thought to be important for prion propagation such as high glutamine/asparagine content are preserved (52, 53, 129, 156, 157). Moreover, these domains can support prion states when expressed in a heterologous *S. cerevisiae* system (52, 53, 129) and in one case examined, in the original yeast species (*Kluyveromyces lactis*) from which it was derived (156). The conservation of the prion-forming abilities of Sup35p together with the observation that presence of the prion can provide a selective advantage in certain conditions (15, 171, 172) suggests that rather than being a pathogen, [*PSI*$^+$] may represent a beneficial and conserved epigenetic mechanism for regulating protein function.

Analogous to the mammalian species barrier which limits induction and transmission, [*PSI*$^+$] prions are typically species specific (52, 53, 129, 156, 157). A particularly robust barrier exists between [*PSI*$^+$] prions formed from *S. cerevisiae* and *Candida albicans* derived *SUP35* prion domains. Although these organisms would not naturally interact, species specificity can be studied using genetically manipulated yeast. Overexpression of *S. cerevisiae* Sup35p induces [*PSI*$^+$] in wildtype *S. cerevisiae* but not

in yeast where the *SUP35* gene encodes for the *C. albicans* prion domain and vice versa (52). Even a single point mutation within the *S. cerevisiae SUP35* sequence is sufficient to confer specificity (65). However, in other cases, cross transmission between different *SUP35* sequences is possible albeit with reduced efficiency (53, 156). Such cross transmission could arise directly from some propensity of those Sup35p to be recruited into heterologous prions or indirectly through interactions with cellular machinery such as chaperones.

Specificity of transmission in other yeast prions

Barriers to transmission between different yeast prions have also been observed. For example overexpression of New1p induces [*NU*$^+$] but not [*PSI*$^+$], whereas overexpression of Sup35p induces [*PSI*$^+$] but not [*NU*$^+$] (49) or [*URE3*]. Finally, transient expression of heterologous species of Ure2p rarely induced [*URE3*] formation in *S. cerevisiae* even though similar expression of the *S. cerevisiae* Ure2p generated [*URE3*]-containing cells (173, 174). However, an important caveat is that it has yet been shown that these alternate species of Ure2p can even form self-propagating prion states. If they cannot, then the lack of induction can be easily explained by the inability to form any type of infectious particles, rather than reflecting a specific transmission barrier between prions.

Antagonism and cooperation between yeast prions

Even when a barrier prevents transmission of prion states between two different prion proteins, the presence of one prion can strongly influence both induction and propagation of a second. This influence can be positive such as in the well-characterized [*PSI*$^+$]-inducibility, or [*PIN*$^+$], effect where *de novo* induction of [*PSI*$^+$] by Sup35p overexpression only occurs in yeast harboring a second prion (49, 131, 132). Alternatively, prions can interfere with each other's propagation. For example, the [*URE3*] state is not inherited stably in [*PSI*$^+$] cells and vice versa (132, 175). The molecular bases of the above phenomena are poorly understood. In particular, a major open question is the extent to which this represents mixed polymers or an indirect effect, such as modulation of aggregation by chaperones.

On a related note, this effect of protein aggregates affecting *de novo* appearance of other aggregates seems to be a general effect, at least in yeast. Recent experiments demonstrated that aggregation of polyglutamine proteins is sensitive to the presence of other yeast prions (49), even though the polyglutamine proteins themselves cannot support prion inheritance in yeast. A mutant allele of the Machado-Joseph Disease (MJD) protein containing an expanded polyglutamine tract fused to GFP was used as a fluorescent reporter of aggregation. Aggregates of the Rnq1p or New1p prion domain were sufficient to promote aggregation of the mutant MJD protein, while in the absence of these aggregates the reporter construct remained soluble (49).

In vitro evidence for a molecular basis of yeast prion specificity

Complementing *in vivo* observations of yeast prion specificity, it has been possible to recapitulate the sequence-specific propagation of the [*PSI*$^+$] prion *in vitro*. Extracts of [*PSI*$^+$] cells expressing *S. cerevisae* Sup35p can induce aggregation of Sup35p present in [*psi*$^-$] extracts from cells expressing *S. cerevisiae* but not *Pichia methanolica* Sup35p (129). An obligatory role for other cellular factors can be eliminated using an *in vitro* polymerization reaction with only purified recombinant prion domains (62, 176). Both *S. cerevisiae* and *C. albicans* derived prion domains form amyloid fibers after characteristic lag times; addition of preformed fibers of *S. cerevisiae* Sup35p prion domains efficiently seeds polymerization of *S. cerevisiae* Sup35p prion domains but not domains derived from *C. albicans* and vice versa. Remarkably, even when present together in a mixture, these two species of prion domains show exquisite sequence specificity and form homopolymeric fibers (52).

Sequence specific amyloid propagation

The sequence specificity seen in the purified Sup35p amyloid system is a common property of amyloid fibers, even of those not involved in prion phenomenon. Recent work with polyglutamine-containing proteins showed that formation of detergent-resistant amyloid aggregates is highly protein specific with coaggregation limited to proteins that share sequence homology outside the polyglutamine tract region (177). Peptides derived from the PrP protein also show preferential formation of homogeneous amyloids (178, 179) and polymers of Aβ have stereochemical specificity for aggregate

formation (180). Finally, *in vivo* specificity is also seen during inclusion body formation and aggresome assembly (181, 182).

RELATIONSHIP BETWEEN PRION STRAINS AND SPECIES BARRIERS

The phenomenological connection between strains and species barrier has long been appreciated. Even before the identification of a infectious agent responsible for transmissible spongiform encephalopathies (TSEs), it was known that scrapie strains played a strong role in determining specificity of transmission (155, 160, 183). Indeed, these observations led to the concept of a 'transmission barrier', rather than a species barrier, to reflect the role of features other than simple sequence homology in determining prion infectivity (2). Understanding this relationship between prion strains and interspecies transmission has become especially relevant with the finding that the recent appearance of new variant CJD (nvCJD) seems to have resulted from the transmission of the prion strain responsible for Mad Cow Disease / bovine spongiform encephalopathy (BSE). BSE appears to be an especially promiscuous prion type, capable of crossing the species barrier that normally prevents transmission of animal prions, such as scrapie, to humans (2, 32, 184-186). Remarkably, the link between strains and species barriers seems to be general as strain variants of yeast prions can differ widely in specificity of transmission (139, 140, 187, 188). Below, we review the evidence linking strains, species barriers and protein conformational changes in various prion systems. A

synthesis of these observations suggest a model in which prion strains and transmission barriers are in large part manifestations of the same phenomenon: the ability of proteins to misfold into multiple amyloid-like conformations. This model helps explain several characteristic features of prion strains and species barriers.

Prion strains affect interspecies mammalian prion transmission

During early studies of TSE infectivity, isolates of sheep scrapie were found to vary in their ability to infect goats, mice and other laboratory animals (189). In one case, two sheep prion strains were investigated, a clinical isolate of naturally occurring sheep scrapie and a strain generated through experimental passage of BSE through sheep. The results were striking: successful transmission of sheep scrapie to laboratory mice took approximately 800 days while the mice inoculated with sheep-passaged BSE strains showed clinical signs in half that time (160). Later these experiments were refined through the use of isogenic transgenic mice. For instance, a transgenic mouse expressing a chimeric human-mouse PrP showed different susceptibility to two hamster-derived prion strains, even though they were composed of the same prion protein (190). Altogether these data argue that the nature of the prion strain is a key component of determining transmission across a species barrier.

Passage through a species barrier modulates prion strains

The relationship between strains and species barrier is reciprocal: just as strains show differing ability to cross between species, crossing a species barrier can result in a shift in strain characteristics. For example, clinical features and pathological hallmarks of scrapie were altered upon inoculation of goats with sheep scrapie. However, the infection of other sheep did not show this shift in scrapie disease profile (191). In studies of transmissible mink encephalopathies, researchers found that transmission of mink-derived 'drowsy' prion strains into hamsters resulted in formation of both 'drowsy' and 'hyper' prion strains in a titer-dependent fashion[Bartz, 2000 #394}. Polymorphisms in the PrP gene present in a single species can also modulate the transmission of prion strains. Passage of BSE through transgenic mice expressing human PrP homozygous for valine at codon 129 does not affect strain type (165, 185). In contrast, BSE transmission to mice expressing human PrP homozygous for methionine at codon 129 resulted in mixture of both the parental BSE/nvCJD strain and new types similar to spontaneous CJD (192). An important caveat is that the existence of a species barrier does not necessitate a change in strain type. For example, in one study, large species barrier effects were observed upon mouse-to-hamster and upon mouse-to-rat transmission when using a particular mouse-derived prion strain. When the material was then inoculated into mice, the mouse-to-hamster passaged strain showed significantly different properties as compared to the parent, but the mouse-to-rat isolate appeared unchanged (191).

Emergence of prion strains is accompanied by a change in conformation

Experiments by Peretz and colleagues helped define a molecular mechanism for the link between species barriers and strains by showing that emergence of new prion

strains following interspecies transmission is accompanied by changes in prion conformations (190). These studies used two hamster prion strains, drowsy (DY) and Sc237, which could be distinguished by relative stability as measured by chemical denaturation (Figure 7) (26). The prion strains were administered to a line of transgenic mice where a chimeric hamster/mouse PrP gene replaced the wildtype mouse allele. Inoculation with the DY strain resulted in rapid onset of disease and a characteristic DY-specific clinical phenotype. Consistent with this observation, the newly converted host PrP^{SC} retained the conformational stability associated with the parent strain. In contrast, the Sc237 hamster prion strain exhibited a delayed transmission characteristic of a species barrier. The passage resulted in the emergence of a strain with significantly different clinical features and conformational stability than the original Sc237 strain. The new strain propagated faithfully in transgenic mice with a fixed period of latency distinct from the Sc237 strain (190). Thus both clinical features and conformational hallmarks of a prion strain can change upon transmission across a species barrier.

Yeast prion strains and sequence-dependent transmission

A number of *in vivo* and *in vitro* experiments have pointed to an intimate link among strains, sequence, and conformational differences in the [*PSI*$^+$] prion systems. An early example of this came from studies of a Sup35p mutant (glycine at residue 58 to aspartic acid - known as PNM2 (193, 194)), which in some contexts is defective in yeast prion propagation (188). As mentioned previously, weak [*PSI*$^+$] variants exhibit mitotic instability and have lower levels of termination suppression when compared to strong

[*PSI*$^+$] variants. Paradoxically, expression of PNM2 interfered with the suppression phenotype of strong [*PSI*$^+$] strains, while expression of PNM2 in a weak [*PSI*$^+$] strain actually enhanced the suppression phenotype (188). As both weak and strong [*PSI*$^+$] variants were in genetically identical backgrounds, the clearest interpretation was that the variants consisted of distinct propagating forms of Sup35p that could be differentially influenced by expression of the mutant protein.

Elegant experiments by C.Y. King (187) further investigated this link between yeast prion strains and sequence-specificity. King explored the ability of three different [*PSI*$^+$] variants to recruit a panel of Sup35p mutants, as monitored by both suppression phenotype and by recruitment of GFP fusions. He found that certain mutants could be preferentially recruited by some of the variants, while other mutants could not be recruited by any of the variants. Furthermore, coexpression of mutant prion domains cured the [*PSI*$^+$] variants to different degrees. These data led to the conclusion that the [*PSI*$^+$] strain variants were due to structurally different Sup35p aggregates, each exposing different regions of the polypeptide. The ability to interact with a mutant would then be determined by the surface presented by a particular aggregate.

Work from our own lab, using a combination of *in vivo* and *in vitro* studies, has directly established that a single polypeptide can form more than one self-propagating amyloid conformation and that these conformations can determine the specificity of prion propagation. Moreover, we demonstrated that point mutations in a prion protein, by changing the spectrum of favored conformations, generate a *de novo* species barrier (139, 140). These experiments used a chimeric prion domain (known as Ch) composed of the

first forty amino acids of the *S. cerevisiae* Sup35p fused to the remainder of the prion domain from *C. albicans*. Whereas a barrier normally inhibits transmission between *S. cerevisiae* and *C. albicans SUP35*, the Ch prion domain is able to bridge this barrier. *In vivo*, Ch formed distinct prion strains with markedly different strengths and specificities upon induction by different Sup35p species (Figure 7). Similarly, when seeded with different species of Sup35p fibers *in vitro*, the purified Ch protein forms two distinct self-propagating amyloid forms. These conformations dictate seeding specificity: Ch seeded by *S. cerevisiae* Sup35p fibers efficiently catalyses conversion of *S. cerevisiae* Sup35p (Sc) but not *C. albicans* Sup35p (Ca), and vice versa (139). These observations indicated that Ch bridges the species barrier by adopting two conformations (Figure 8) – one that is specific for Sc (Ch[Sc]) and the other specific for Ca (Ch[Ca]).

This work was extended by looking at the effect of mutations in the Ch protein that were chosen to specifically disfavor Ch[Sc] or Ch[Ca]. Mutations which inhibited formation of the Ch[Ca] state, both *in vivo* and *in vitro,* prevented transmission between Ch and Ca without disrupting transmission to Sc. Conversely, mutants disfavoring Ch[Sc] were incapable of transmitting to Sc, but remained susceptible to Ca. Interestingly, modulation of temperature also strongly influenced the preference for forming Ch[Sc] and Ch[Ca] (140). These observations indicate how changes in sequence of a prion or the changes in environment can affect a prion's specificity by modulating conformations.

MODEL INTEGRATING PRION STRAINS, SPECIES BARRIERS AND PRINCIPLES OF AMYLOID FORMATION

Tenets of the model

A synthesis of the experimental observations above suggests the following tenets linking prion strains, species barriers and the physical principles that govern protein misfolding. For the most part, these tenets have substantial experimental support and can also serve to guide the direction of future experiments.

1. Self-propagation of amyloid-like protein aggregates underlies prion growth.
2. A single protein can often misfold into multiple different amyloid conformations.
3. The phenotypic consequences resulting from an aggregated protein are highly dependent on the specific amyloid conformation.
4. The particular amyloid conformation that a protein adopts determines the specificity of growth.
5. Changes in protein sequence can modulate the spectrum of favored amyloid conformations.

Relationship between conformation, strains and species barriers

Based on these tenets, a model emerges in which prion strains and transmission barriers are in large part two different manifestations of the same phenomenon, the ability

of a protein to misfold into multiple amyloid conformations. These conformations in turn determine both the specificity of growth and the phenotypic consequences of harboring a prion. Changes in sequence alter the range of preferred amyloid conformations thereby modulating transmission barriers and strain phenotypes.

Though this model, based on the propagation of amyloid-like structures, can account for many observed prion phenomena, it is clear that host cellular factors such as chaperones or degradative machinery can play a significant role in both the phenotype and propagation of prions. These factors are also likely to contribute to strains and species barriers by mechanisms other than changes in conformation. Furthermore, the simple ability to form a self-propagating aggregated state does not guarantee that a protein will be infectious. While amyloid-like aggregation forms a physical basis for the propagation of prions, true understanding of what makes a prion more than an aggregated protein remains a central challenge. Nonetheless, our model suggests explanations for several features of prion inheritance.

Strains are a common feature of prion inheritance Extensive evidence from both mammalian and yeast prion systems show that different propagating amyloid-like conformations are strongly correlated with distinct prion strains. This may be a specific case of the more general ability of amyloid fibers to form a range of self-propagating conformations. The ability of each conformation to robustly propagate differences such as distinct fiber morphologies and assembly kinetics could lead directly to heritable variation in phenotypes. For example, if the phenotype is due to the amount of soluble

protein, variations in the aggregation rate will directly influence phenotype. Alternately, cellular factors may interact differently with the various conformations, leading to a distinct physiological outcome for different prion aggregates. A major goal is to elucidate the mechanism by which alternate prion conformations can cause different strain phenotypes.

Transmission barriers are common and apparently easy to generate Not only are species-specific transmission barriers a ubiquitous feature of prion propagation, they also arise rapidly, as evidenced by the small number of amino acid changes required to inhibit transmission between prions. This phenomenon can be explained by the ability of changes in polypeptide primary structure to alter the range of preferred amyloid fiber conformations. Even single point mutations can in some cases shift the fiber conformation, resulting in a novel self-specific aggregate that is incompatible with the original parent sequence.

Strains determine transmission specificity Because amyloid conformations vary in their ability to recruit heterologous proteins, prion particles composed of the same protein but differing in their strain conformation will differ in their compatibility with the corresponding prion protein from another species. Variation in conformational compatibility between prion proteins thereby contributes to strain-specific transmission across a species barrier.

Strains switch upon transmission across a species barrier Crossing a species barrier, though inefficient, is possible if a compatible prion conformation can be found and amplified. Observed switches in strains therefore may result from such an amplification of a conformation compatible with cross-species transmission. Although there are a variety of models which may explain this effect, two major possibilities stand out (2, 190) (Figure 9). One, upon interspecies passage, assembly of the new polypeptide onto the infectious seed results in a new conformation. Two, new protein selectively grows on the subset of compatible seeds. In this interpretation, the transmission barrier acts as a sieve – by selectively amplifying one component of a pool of conformations. This model demands that the initial strain actually consist of a number of subtypes, and that the biological strain phenotype reflects this collection.

PERSPECTIVE

The ability of proteins to adopt multiple amyloid forms indicates a fundamental difference between the rules of protein folding and misfolding. Globular folds are stabilized by multiple cooperative interactions between specific sidechains resulting in unique well-defined structures. By contrast, recent studies indicate that amyloid formation is driven predominantly by mainchain interactions, which can be locally favored or disfavored by specific sidechains (195). As a consequence, a polypeptide can adopt multiple amyloid forms differing in their quaternary or possibly tertiary structures, with specific sidechains disfavoring a particular subset of structures without preventing amyloid formation altogether. Small differences in the rates of forming the various conformations will be reinforced by the self-propagating nature of amyloid formation;

once a stable nucleus of a given conformation is formed it rapidly dominate the reaction as it grows exponentially. Because of this, the reaction will be under kinetic control, with the final conformation choice determined by the specific conditions of polymerization rather than the global thermodynamic minimum.

The fact that one polypeptide can misfold into multiple self-propagating forms helps explain a range of observations regarding prion inheritance. For example, the existence of transmission barriers between highly related species can be explained by the fact that the infectious conformation is sensitive to small changes in primary structure. Mutations affect the initial choice of conformation during *de novo* prion formation and in turn the conformation of the prion will determine which sequences can be recruited. A robust transmission barrier will therefore arise when the range of conformations adopted by two sequences are incompatible (2, 52, 53, 129, 156). Similarly, if the conformation affects phenotype as well as specificity, then changes in the primary structure of a prion protein could also alter the strain phenotype by shifting the infectious conformations (161, 168). It has been reported that crossing a transmission barrier can result in a change in the prion strain. Because conformations vary in their specificity, the transmission barrier could act as a sieve, selectively amplifying infectious forms compatible with the recipient prion sequence(2, 190). Finally, the failure to create transmissible forms of the mammalian prion protein (PrP) *in vitro* despite a number of reports demonstrating production of self-propagating or protease resistant PrP states (196-198) could be due to the preferential formation of non-infectious conformations outside the normal cellular context.

The degeneracy of amyloid formation may be important for understanding a range of protein misfolding disorders. There is increasing evidence that the toxicity of the different misfolded forms varies greatly, with some species being highly pathogenic while others might even be protective (107, 109, 111). Given the strong propensity of non-native proteins to aggregate, therapeutic strategies designed to promote formation of non-toxic conformations rather than preventing amyloid formation altogether may be more tractable. In addition to selective pressure for function, this variability suggests that polypeptide sequences that form less toxic conformations when they do misfold will be preferred. More generally, any analysis of formation and consequences of amyloid-like aggregates needs to be tempered with the knowledge there exists a range of conformationally distinct subtypes that will influence their biological effects.

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1. Prusiner SB, Scott MR, DeArmond SJ, Cohen FE. 1998. *Cell* 93: 337-48

2. Collinge J. 2001. *Annu Rev Neurosci* 24: 519-50

3. Cohen FE, Prusiner SB. 1998. *Annu Rev Biochem* 67: 793-819

4. Horwich AL, Weissman JS. 1997. *Cell* 89: 499-510

5. Griffith JS. 1967. *Nature* 215: 1043-4

6. Roberts BT, Wickner RB. 2003. *Genes Dev*

7. Priola SA, Chesebro B, Caughey B. 2003. *Science* 300: 917-9

8. Bolton DC, McKinley MP, Prusiner SB. 1982. *Science* 218: 1309-11

9. Lasmezas CI, Deslys JP, Robain O, Jaegly A, Beringue V, et al. 1997. *Science* 275: 402-5

10. Deleault NR, Lucassen RW, Supattapone S. 2003. *Nature* 425: 717

11. Wickner RB. 1994. *Science* 264: 566-9

12. Wickner RB, Taylor KL, Edskes HK, Maddelein ML, Moriyama H, Roberts BT. 2001. *Adv Protein Chem* 57: 313-34

13. Serio TR, Lindquist SL. 2001. *Adv Protein Chem* 57: 335-66

14. Uptain SM, Lindquist S. 2002. *Annu Rev Microbiol* 56: 703-41

15. Osherovich LZ, Weissman JS. 2002. *Dev Cell* 2: 143-51

16. Sparrer HE, Santoso A, Szoka FC, Weissman JS. 2000. *Science* 289: 595-9

17. Maddelein ML, Dos Reis S, Duvezin-Caubet S, Coulary-Salin B, Saupe SJ. 2002. *Proc Natl Acad Sci U S A* 99: 7402-7

18. Dobson CM. 1999. *Trends in Biochemical Sciences* 24: 329-32

19. Prusiner SB, Bolton DC, Groth DF, Bowman KA, Cochran SP, McKinley MP. 1982. *Biochemistry* 21: 6942-50

20. Prusiner SB, Groth DF, Bolton DC, Kent SB, Hood LE. 1984. *Cell* 38: 127-34

21. Oesch B, Westaway D, Walchli M, McKinley MP, Kent SB, et al. 1985. *Cell* 40: 735-46

22. Chesebro B, Race R, Wehrly K, Nishio J, Bloom M, et al. 1985. *Nature* 315: 331-3

23. Stahl N, Baldwin MA, Teplow DB, Hood L, Gibson BW, et al. 1993. *Biochemistry* 32: 1991-2002

24. Baldwin MA. 2001. *Adv Protein Chem* 57: 29-54

25. Peretz D, Williamson RA, Matsunaga Y, Serban H, Pinilla C, et al. 1997. *J Mol Biol* 273: 614-22

26. Peretz D, Scott MR, Groth D, Williamson RA, Burton DR, et al. 2001. *Protein Sci* 10: 854-63.

27. James TL, Liu H, Ulyanov NB, Farr-Jones S, Zhang H, et al. 1997. *Proc Natl Acad Sci U S A* 94: 10086-91

28. Hosszu LL, Baxter NJ, Jackson GS, Power A, Clarke AR, et al. 1999. *Nat Struct Biol* 6: 740-3

29. Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K. 1996. *Nature* 382: 180-2

30. Lopez Garcia F, Zahn R, Riek R, Wuthrich K. 2000. *Proc Natl Acad Sci U S A* 97: 8334-9

31. Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, et al. 1993. *Proc Natl Acad Sci U S A* 90: 10962-6

32. Prusiner SB. 1998. *Proceedings of the National Academy of Sciences of the United States of America* 95: 13363-83

33. Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, et al. 1993. *Cell* 73: 1339-47

34. Prusiner SB, Groth D, Serban A, Koehler R, Foster D, et al. 1993. *Proc Natl Acad Sci U S A* 90: 10608-12

35. Bueler H, Raeber A, Sailer A, Fischer M, Aguzzi A, Weissmann C. 1994. *Mol Med* 1: 19-30

36. Carlson GA, Ebeling C, Yang SL, Telling G, Torchia M, et al. 1994. *Proc Natl Acad Sci U S A* 91: 5690-4

37. Manson JC, Clarke AR, McBride PA, McConnell I, Hope J. 1994. *Neurodegeneration* 3: 331-40

38. Enari M, Flechsig E, Weissmann C. 2001. *Proc Natl Acad Sci U S A* 98: 9295-9

39. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, et al. 1994. *Nature* 370: 471-4

40. Kocisko DA, Priola SA, Raymond GJ, Chesebro B, Lansbury PT, Jr., Caughey B. 1995. *Proceedings of the National Academy of Sciences of the United States of America* 92: 3923-7

41. Saborio GP, Permanne B, Soto C. 2001. *Nature* 411: 810-3

42. Lucassen R, Nishina K, Supattapone S. 2003. *Biochemistry* 42: 4127-35

43. Wille H, Zhang GF, Baldwin MA, Cohen FE, Prusiner SB. 1996. *J Mol Biol* 259: 608-21

44. Tzaban S, Friedlander G, Schonberger O, Horonchik L, Yedidia Y, et al. 2002. *Biochemistry* 41: 12868-75

45. Masison DC, Wickner RB. 1995. *Science* 270: 93-5

46. Patino MM, Liu JJ, Glover JR, Lindquist S. 1996. *Science* 273: 622-6

47. Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. 1996. *Embo J* 15: 3127-34

48. Schlumpberger M, Prusiner SB, Herskowitz I. 2001. *Mol Cell Biol* 21: 7035-46

49. Osherovich LZ, Weissman JS. 2001. *Cell* 106: 183-94

50. Sondheimer N, Lindquist S. 2000. *Mol Cell* 5: 163-72

51. Coustou V, Deleu C, Saupe S, Begueret J. 1997. *Proc Natl Acad Sci U S A* 94: 9773-8

52. Santoso A, Chien P, Osherovich LZ, Weissman JS. 2000. *Cell* 100: 277-88

53. Chernoff YO, Galkin AP, Lewitin E, Chernova TA, Newnam GP, Belenkiy SM. 2000. *Mol Microbiol* 35: 865-76

54. Fernandez-Bellot E, Guillemet E, Cullin C. 2000. *Embo J* 19: 3215-22

55. Fernandez-Bellot E, Guillemet E, Ness F, Baudin-Baillieu A, Ripaud L, et al. 2002. *EMBO Rep* 3: 76-81

56. Coustou-Linares V, Maddelein ML, Begueret J, Saupe SJ. 2001. *Mol Microbiol* 42: 1325-35

57. Zhou P, Derkatch IL, Liebman SW. 2001. *Mol Microbiol* 39: 37-46

58. Ripaud L, Maillet L, Cullin C. 2003. *Embo J* 22: 5251-9

59. Kimura Y, Koitabashi S, Fujita T. 2003. *Cell Struct Funct* 28: 187-93

60. Speransky VV, Taylor KL, Edskes HK, Wickner RB, Steven AC. 2001. *J Cell Biol* 153: 1327-36

61. Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. 1997. *Science* 277: 381-3

62. Glover JR, Kowal AS, Schirmer EC, Patino MM, Liu JJ, Lindquist S. 1997. *Cell* 89: 811-9

63. Taylor KL, Cheng N, Williams RW, Steven AC, Wickner RB. 1999. *Science* 283: 1339-43

64. Dos Reis S, Coulary-Salin B, Forge V, Lascu I, Begueret J, Saupe SJ. 2002. *J Biol Chem* 277: 5703-6

65. DePace AH, Santoso A, Hillner P, Weissman JS. 1998. *Cell* 93: 1241-52

66. Li L, Lindquist S. 2000. *Science* 287: 661-4

67. Harper JD, Lansbury PT, Jr. 1997. *Annu Rev Biochem* 66: 385-407

68. Rochet JC, Lansbury PT, Jr. 2000. *Curr Opin Struct Biol* 10: 60-8

69. Thirumalai D, Klimov DK, Dima RI. 2003. *Curr Opin Struct Biol* 13: 146-59

70. Masel J, Jansen VA. 1999. *Proc R Soc Lond B Biol Sci* 266: 1927-31

71. Serio TR, Cashikar AG, Kowal AS, Sawicki GJ, Moslehi JJ, et al. 2000. *Science* 289: 1317-21

72. Scheibel T, Lindquist SL. 2001. *Nat Struct Biol* 8: 958-62

73. Padrick SB, Miranker AD. 2002. *Biochemistry* 41: 4694-703

74. Chen S, Berthelier V, Hamilton JB, O'Nuallain B, Wetzel R. 2002. *Biochemistry* 41: 7391-9

75. Taylor JP, Hardy J, Fischbeck KH. 2002. *Science* 296: 1991-5

76. Kelly JW. 1996. *Curr Opin Struct Biol* 6: 11-7

77. Chiti F, Webster P, Taddei N, Clark A, Stefani M, et al. 1999. *Proc Natl Acad Sci U S A* 96: 3590-4

78. Guijarro JI, Sunde M, Jones JA, Campbell ID, Dobson CM. 1998. *Proc Natl Acad Sci U S A* 95: 4224-8

79. Sunde M, Serpell LC, Bartlam M, Fraser PE, Pepys MB, Blake CC. 1997. *J Mol Biol* 273: 729-39

80. Perutz MF, Finch JT, Berriman J, Lesk A. 2002. *Proc Natl Acad Sci U S A* 99: 5591-5

81. Wetzel R. 2002. *Structure (Camb)* 10: 1031

82. Serpell LC, Sunde M, Benson MD, Tennent GA, Pepys MB, Fraser PE. 2000. *Journal of Molecular Biology* 300: 1033-9

83. Jimenez JL, Guijarro JI, Orlova E, Zurdo J, Dobson CM, et al. 1999. *Embo J* 18: 815-21

84. Richardson JS, Richardson DC. 2002. *Proc Natl Acad Sci U S A* 99: 2754-9

85. Prusiner SB, McKinley MP, Bowman KA, Bolton DC, Bendheim PE, et al. 1983. *Cell* 35: 349-58

86. Wille H, Michelitsch MD, Guenebaut V, Supattapone S, Serban A, et al. 2002. *Proc Natl Acad Sci U S A* 99: 3563-8

87. Knaus KJ, Morillas M, Swietnicki W, Malone M, Surewicz WK, Yee VC. 2001. *Nat Struct Biol* 8: 770-4

88. Bousset L, Briki F, Doucet J, Melki R. 2003. *J Struct Biol* 141: 132-42

89. Baxa U, Taylor KL, Wall JS, Simon MN, Cheng N, et al. 2003. *J Biol Chem*

90. Kayed R, Bernhagen J, Greenfield N, Sweimeh K, Brunner H, et al. 1999. *Journal of Molecular Biology* 287: 781-96

91. Wood SJ, Wypych J, Steavenson S, Louis JC, Citron M, Biere AL. 1999. *J Biol Chem* 274: 19509-12

92. Friedhoff P, von Bergen M, Mandelkow EM, Davies P, Mandelkow E. 1998. *Proc Natl Acad Sci U S A* 95: 15712-7

93. Scherzinger E, Sittler A, Schweiger K, Heiser V, Lurz R, et al. 1999. *Proc Natl Acad Sci U S A* 96: 4604-9

94. Morozova-Roche LA, Zurdo J, Spencer A, Noppe W, Receveur V, et al. 2000. *J Struct Biol* 130: 339-51

95. Selkoe DJ. 1999. *Nature* 399: A23-31

96. Goudsmit J, Morrow CH, Asher DM, Yanagihara RT, Masters CL, et al. 1980. *Neurology* 30: 945-50

97. Godec MS, Asher DM, Kozachuk WE, Masters CL, Rubi JU, et al. 1994. *Neurology* 44: 1111-5

98. Chernoff YO, Derkach IL, Inge-Vechtomov SG. 1993. *Current Genetics* 24: 268-70

99. Yamin G, Glaser CB, Uversky VN, Fink AL. 2003. *J Biol Chem* 278: 27630-5

100. Uversky VN, Li J, Bower K, Fink AL. 2002. *Neurotoxicology* 23: 527-36

101. Chernoff YO, Lindquist SL, Ono B, Inge-Vechtomov SG, Liebman SW. 1995. *Science* 268: 880-4

102. Glover JR, Lindquist S. 1998. *Cell* 94: 73-82

103. Aguzzi A, Heppner FL, Heikenwalder M, Prinz M, Mertz K, et al. 2003. *Br Med Bull* 66: 141-59

104. Borchsenius AS, Wegrzyn RD, Newnam GP, Inge-Vechtomov SG, Chernoff YO. 2001. *Embo J* 20: 6683-91

105. Lundmark K, Westermark GT, Nystrom S, Murphy CL, Solomon A, Westermark P. 2002. *Proc Natl Acad Sci U S A* 99: 6979-84

106. Hill AF, Joiner S, Linehan J, Desbruslais M, Lantos PL, Collinge J. 2000. *Proc Natl Acad Sci U S A* 97: 10248-53.

107. Caughey B, Lansbury PT, Jr. 2003. *Annu Rev Neurosci*

108. Pepys MB, Herbert J, Hutchinson WL, Tennent GA, Lachmann HJ, et al. 2002. *Nature* 417: 254-9

109. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, et al. 2002. *Nature* 416: 535-9

110. Conway KA, Rochet JC, Bieganski RM, Lansbury PT, Jr. 2001. *Science* 294: 1346-9

111. Bucciantini M, Giannoni E, Chiti F, Baroni F, Formigli L, et al. 2002. *Nature* 416: 507-11

112. Ter-Avanesyan MD, Dagkesamanskaya AR, Kushnirov VV, Smirnov VN. 1994. *Genetics* 137: 671-6

113. Derkatch IL, Chernoff YO, Kushnirov VV, Inge-Vechtomov SG, Liebman SW. 1996. *Genetics* 144: 1375-86

114. Bradley ME, Edskes HK, Hong JY, Wickner RB, Liebman SW. 2002. *Proc Natl Acad Sci U S A* 99 Suppl 4: 16392-9

115. Weissmann C. 1991. *Nature* 352: 679-83

116. Pattison I, Millson GC. 1961. *J Comp Pathol* 71: 101-8

117. Fraser H, Dickinson AG. 1968. *J Comp Pathol* 78: 301-11

118. Bruce ME, McConnell I, Fraser H, Dickinson AG. 1991. *J Gen Virol* 72 (Pt 3): 595-603

119. Caughey B, Raymond GJ, Bessen RA. 1998. *Journal of Biological Chemistry* 273: 32230-5

120. Somerville RA, Oberthur RC, Havekost U, MacDonald F, Taylor DM, Dickinson AG. 2002. *J Biol Chem* 277: 11084-9

121. Safar J, Wille H, Itri V, Groth D, Serban H, et al. 1998. *Nature Medicine* 4: 1157-65

122. Priola SA, Lawson VA. 2001. *Embo J* 20: 6692-9

123. Vorberg I, Priola SA. 2002. *J Biol Chem* 277: 36775-81

124. Bessen RA, Marsh RF. 1992. *J Virol* 66: 2096-101

125. Telling GC, Parchi P, DeArmond SJ, Cortelli P, Montagna P, et al. 1996. *Science* 274: 2079-82

126. Bessen RA, Kocisko DA, Raymond GJ, Nandan S, Lansbury PT, Caughey B. 1995. *Nature* 375: 698-700

127. Telling GC, Scott M, Hsiao KK, Foster D, Yang SL, et al. 1994. *Proc Natl Acad Sci U S A* 91: 9936-40

128. Telling GC, Scott M, Mastrianni J, Gabizon R, Torchia M, et al. 1995. *Cell* 83: 79-90

129. Kushnirov VV, Kochneva-Pervukhova NV, Chechenova MB, Frolova NS, Ter-Avanesyan MD. 2000. *Embo J* 19: 324-31

130. Kushnirov VV, Kryndushkin DS, Boguta M, Smirnov VN, Ter-Avanesyan MD. 2000. *Curr Biol* 10: 1443-6

131. Derkatch IL, Bradley ME, Masse SV, Zadorsky SP, Polozkov GV, et al. 2000. *Embo J* 19: 1942-52

132. Derkatch IL, Bradley ME, Hong JY, Liebman SW. 2001. *Cell* 106: 171-82

133. Zhou P, Derkatch IL, Uptain SM, Patino MM, Lindquist S, Liebman SW. 1999. *Embo J* 18: 1182-91

134. Kochneva-Pervukhova NV, Chechenova MB, Valouev IA, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. 2001. *Yeast* 18: 489-97

135. All-Robyn JA, Kelley-Geraghty D, Griffin E, Brown N, Liebman SW. 1990. *Genetics* 124: 505-14

136. Uptain SM, Sawicki GJ, Caughey B, Lindquist S. 2001. *Embo J* 20: 6236-45

137. Baxa U, Speransky V, Steven AC, Wickner RB. 2002. *Proc Natl Acad Sci U S A* 99: 5253-60

138. DePace AH, Weissman JS. 2002. *Nat Struct Biol* 9: 389-96

139. Chien P, Weissman JS. 2001. *Nature* 410: 223-7

140. Chien P, DePace AH, Collins SR, Weissman JS. 2003. *Nature* 424: 948-51

141. Kirschner DA, Inouye H, Duffy LK, Sinclair A, Lind M, Selkoe DJ. 1987. *Proc Natl Acad Sci U S A* 84: 6953-7

142. Halverson K, Fraser PE, Kirschner DA, Lansbury PT, Jr. 1990. *Biochemistry* 29: 2639-44

143. Goldsbury CS, Cooper GJ, Goldie KN, Muller SA, Saafi EL, et al. 1997. *J Struct Biol* 119: 17-27

144. Goldsbury C, Goldie K, Pellaud J, Seelig J, Frey P, et al. 2000. *J Struct Biol* 130: 352-62

145. Kad NM, Thomson NH, Smith DP, Smith DA, Radford SE. 2001. *J Mol Biol* 313: 559-71.

146. Zurdo J, Guijarro JI, Jimenez JL, Saibil HR, Dobson CM. 2001. *J Mol Biol* 311: 325-40.

147. Abe H, Kawasaki K, Nakanishi H. 2002. *J Biochem (Tokyo)* 132: 863-74

148. Miravalle L, Tokuda T, Chiarle R, Giaccone G, Bugiani O, et al. 2000. *J Biol Chem* 275: 27110-6

149. Fraser PE, Duffy LK, O'Malley MB, Nguyen J, Inouye H, Kirschner DA. 1991. *J Neurosci Res* 28: 474-85

150. Fraser PE, McLachlan DR, Surewicz WK, Mizzen CA, Snow AD, et al. 1994. *J Mol Biol* 244: 64-73

151. Helms LR, Wetzel R. 1996. *J Mol Biol* 257: 77-86

152. Li J, Uversky VN, Fink AL. 2001. *Biochemistry* 40: 11604-13

153. Lashuel HA, Hartley D, Petre BM, Walz T, Lansbury PT, Jr. 2002. *Nature* 418: 291

154. Li J, Uversky VN, Fink AL. 2002. *Neurotoxicology* 23: 553-67

155. Pattison IH. 1965. *Slow, Latent, and Temperature Virus Infections, NINDB Monograph2, D.C Gadjusek, C.J. Gibbs, Jr., and M.P. Alpers, eds. (Washington, D.C.: U.S. Government Printing)*: 249-57

156. Nakayashiki T, Ebihara K, Bannai H, Nakamura Y. 2001. *Mol Cell* 7: 1121-30

157. Resende C, Parham SN, Tinsley C, Ferreira P, Duarte JA, Tuite MF. 2002. *Microbiology* 148: 1049-60

158. Scott M, Foster D, Mirenda C, Serban D, Coufal F, et al. 1989. *Cell* 59: 847-57

159. Prusiner SB, Scott M, Foster D, Pan KM, Groth D, et al. 1990. *Cell* 63: 673-86

160. Bruce M, Chree A, McConnell I, Foster J, Pearson G, Fraser H. 1994. *Philos Trans R Soc Lond B Biol Sci* 343: 405-11

161. Mastrianni JA, Capellari S, Telling GC, Han D, Bosque P, et al. 2001. *Neurology* 57: 2198-205

162. Manson JC, Jamieson E, Baybutt H, Tuzi NL, Barron R, et al. 1999. *Embo J* 18: 6855-64

163. Manson JC, Barron R, Jamieson E, Baybutt H, Tuzi N, et al. 2000. *Arch Virol Suppl*: 95-102

164. Bossers A, Belt P, Raymond GJ, Caughey B, de Vries R, Smits MA. 1997. *Proc Natl Acad Sci U S A* 94: 4931-6

165. Collinge J, Palmer MS, Sidle KC, Hill AF, Gowland I, et al. 1995. *Nature* 378: 779-83

166. Supattapone S, Bosque P, Muramoto T, Wille H, Aagaard C, et al. 1999. *Cell* 96: 869-78

167. Scott MR, Safar J, Telling G, Nguyen O, Groth D, et al. 1997. *Proc Natl Acad Sci U S A* 94: 14279-84

168. Barron RM, Thomson V, Jamieson E, Melton DW, Ironside J, et al. 2001. *Embo J* 20: 5070-8.

169. Priola SA, Chesebro B. 1995. *J Virol* 69: 7754-8

170. Raymond GJ, Bossers A, Raymond LD, O'Rourke KI, McHolland LE, et al. 2000. *Embo J* 19: 4425-30

171. True HL, Lindquist SL. 2000. *Nature* 407: 477-83

172. Eaglestone SS, Cox BS, Tuite MF. 1999. *Embo J* 18: 1974-81

173. Baudin-Baillieu A, Fernandez-Bellot E, Reine F, Coissac E, Cullin C. 2003. *Mol Biol Cell* 14: 3449-58

174. Edskes HK, Wickner RB. 2002. *Proc Natl Acad Sci U S A* 99 Suppl 4: 16384-91

175. Schwimmer C, Masison DC. 2002. *Mol Cell Biol* 22: 3590-8

176. King CY, Tittmann P, Gross H, Gebert R, Aebi M, Wuthrich K. 1997. *Proc Natl Acad Sci U S A* 94: 6618-22

177. Busch A, Engemann S, Lurz R, Okazawa H, Lehrach H, Wanker EE. 2003. *J Biol Chem*

178. Come JH, Fraser PE, Lansbury PT, Jr. 1993. *Proc Natl Acad Sci U S A* 90: 5959-63

179. Kundu B, Maiti NR, Jones EM, Surewicz KA, Vanik DL, Surewicz WK. 2003. *Proc Natl Acad Sci U S A*

180. Esler WP, Stimson ER, Fishman JB, Ghilardi JR, Vinters HV, et al. 1999. *Biopolymers* 49: 505-14

181. Speed MA, Wang DI, King J. 1996. *Nat Biotechnol* 14: 1283-7

182. Rajan RS, Illing ME, Bence NF, Kopito RR. 2001. *Proc Natl Acad Sci U S A* 98: 13060-5

183. Dickinson AG, Fraser H. 1969. *Nature* 222: 892-3

184. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. 1996. *Nature* 383: 685-90

185. Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, et al. 1997. *Nature* 389: 448-50, 526

186. Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, et al. 1996. *Lancet* 347: 921-5

187. King CY. 2001. *J Mol Biol* 307: 1247-60

188. Derkatch IL, Bradley ME, Zhou P, Liebman SW. 1999. *Curr Genet* 35: 59-67

189. Zlotnick I. 1965. *Slow, Latent, and Temperature Virus Infections, NINDB Monograph2, D.C Gadjusek, C.J. Gibbs, Jr., and M.P. Alpers, eds. (Washington, D.C.: U.S. Government Printing)*: 237-48

190. Peretz D, Williamson RA, Legname G, Matsunaga Y, Vergara J, et al. 2002. *Neuron* 34: 921-32.

191. Kimberlin RH, Cole S, Walker CA. 1987. *J Gen Virol* 68 (Pt 7): 1875-81

192. Asante EA, Linehan JM, Desbruslais M, Joiner S, Gowland I, et al. 2002. *Embo J* 21: 6358-66.

193. Doel SM, McCready SJ, Nierras CR, Cox BS. 1994. *Genetics* 137: 659-70

194. Kochneva-Pervukhova NV, Paushkin SV, Kushnirov VV, Cox BS, Tuite MF, Ter-Avanesyan MD. 1998. *Embo J* 17: 5805-10

195. Fandrich M, Dobson CM. 2002. *Embo J* 21: 5682-90.

196. Hill AF, Antoniou M, Collinge J. 1999. *J Gen Virol* 80: 11-4.

197. Post K, Brown DR, Groschup M, Kretzschmar HA, Riesner D. 2000. *Arch Virol Suppl* 16: 265-73

198. Baskakov IV, Legname G, Baldwin MA, Prusiner SB, Cohen FE. 2002. *J Biol Chem* 277: 21140-8

199. Liu H, Farr-Jones S, Ulyanov NB, Llinas M, Marqusee S, et al. 1999. *Biochemistry* 38: 5362-77

200. Scott MR, Will R, Ironside J, Nguyen HO, Tremblay P, et al. 1999. *Proc Natl Acad Sci U S A* 96: 15137-42

FIGURE LEGENDS

Figure 1 PrP^{C} and PrP^{SC} are conformationally distinct. A, Solution NMR structure of Syrian hamster PrP^{C}, residues 90-231. The structure is predominantly alpha-helical, with an unstructured amino-terminus (199). B, Negative stain EM of Syrian hamster PrP^{27-30} (Sc237 strain), stained with uranyl acetate. The material is in insoluble protease-resistant high molecular weight aggregates that are predominantly β-sheet. Scale bar is 100 nm. Image courtesy of Dr. Holger Wille. C, Summary of differences between PrP^{C} and PrP^{SC}.

Figure 2 The yeast prions [*PSI*$^{+}$] and [*URE3*] are due to self-propagating protein conformations. A, Sup35p is a modular protein involved in translation termination whose self-propagating aggregation is responsible for the [*PSI*$^{+}$] phenotype. The amino-terminal prion forming domain, (N; green), is glutamine and asparagine-rich. The middle domain, (M; blue) is rich in charged residues. The carboxy-terminal domain, (C; orange), contains the essential translation-termination function of the protein. B, Sup35p is soluble in [*psi*$^{-}$] yeast and able to facilitate translation termination while in [*PSI*$^{+}$] yeast, Sup35p is aggregated resulting in suppression of nonsense codons. Translation termination can be monitored using an *ADE1* reporter harboring a premature stop codon. [*PSI*$^{+}$] cells are white and capable of growth on media lacking adenine, while [*psi*$^{-}$] yeast accumulate a red pigment due to lack of Ade1p and are incapable of growth on adenine-less media. C, Ure2p is a modular protein involved in regulation of nitrogen catabolism whose self-propagating aggregation is responsible for the [*URE3*] phenotype. In addition

to the glutamine/asparagines rich amino-terminus (green), Ure2p also contains another region that facilitates prion behavor (green) and portions that antagonize prion formation (black). The remainder of the protein (orange) resembles glutathione-S-transferase and is necessary for Ure2p signaling of the presence of high quality nitrogen sources through Gln3p. D, Normally Ure2p binds the transcription factor Gln3p preventing the upregulation of genes, such as *DAL5*, required for uptake of poor nitrogen sources. Serendipitously, Dal5p imports not only the poor nitrogen source allantoate, but also USA (n-carbamyl aspartate), an intermediate in uracil biosynthesis, thus [*ure-o*] yeast cannot grow on USA medium lacking uracil. In [*URE3*] yeast, Ure2p is aggregated and inactive, leading to constitutive activation of Dal5p and enabling growth on USA media lacking uracil.

Figure 3 Amyloid-like fibers are formed by a variety of prion proteins . A, EM of Syrian hamster $PrP^{27\text{-}30}$ (Sc237 strain), stained with uranyl acetate. Bar = 100nm. Image courtesy of Dr. Holger Wille. B, Amyloid fibers formed by Sup35NM, stained with uranyl acetate. Sup35NM fibers are on average 5 – 10 nm in diameter. C, D, EM of full length Ure2p fibers stained with uranyl acetate before (C) and after (D) digestion with proteinase K. Arrow in D indicates position of a single fiber. Bar = 100 nm (89). E, Amyloid fibers formed by full length Ure2p, stained with vanadate and visualized by dark-field STEM. Arrow indicates the core of the fiber. Bar = 50 nm (89).

Figure 4 Two models for amyloid structure. Both fulfill the requirements of the cross-β fold, where individual β-strands are oriented perpendicular to the fiber axis, whereas β-

sheets are oriented parallel to it. A, Model from cryo-EM studies of amyloid formed by the SH3 domain from PIP_3 kinase (83). B, An example of a left-handed β-helix (from UDP-N-acetylglucosamine pyrophosphorylase of *S. pneumoniae* – PDB ID 1G97), which has been proposed to resemble PrP^{SC} (86). Image courtesy of Dr. Cedric Govaerts.

Figure 5 Steps in prion transmission. A general replication cycle for self-propagating conformationally-based prion protein is shown on the left. Corresponding steps during prion infection in mammals and prion inheritance in fungi are shown on the right.

Figure 6 Amyloid fibers adopt multiple distinguishable structures. A, B, C, Amyloid fibers formed spontaneously by Sup35NM vary in their growth patterns, including overall rate and polarity of growth (138). Four kinetic fiber types visualized by an AFM single fiber growth assay are shown. The original seed is labeled with antibody, and therefore wider than the new growth extending from its ends. Note the presence of long and short symmetric and asymmetric fibers. Scale bar is 500 nm. D, E, F, G, Negative stain EM of amyloid fibers formed spontaneously by the SH3 domain from PIP_3 kinase illustrates that they vary in the number of protofilaments and helical pitch (83). Scale bar is 100 nm.

Figure 7 Strain phenotypes *in vivo* correlate with *in vitro* differences in prion protein. A, Subcallosal plaques caused in transgenic mice expressing bovine *PRNP* characteristic of infection by the indicated prion strain (200). B, Denaturation profile of indicated strains of PrP^{SC} showing stability differences (26). C, Yeast harboring *SUP35* with Ch prion domain, induced to prion state by either Sc (Ch[Sc]) or Ca overexpression

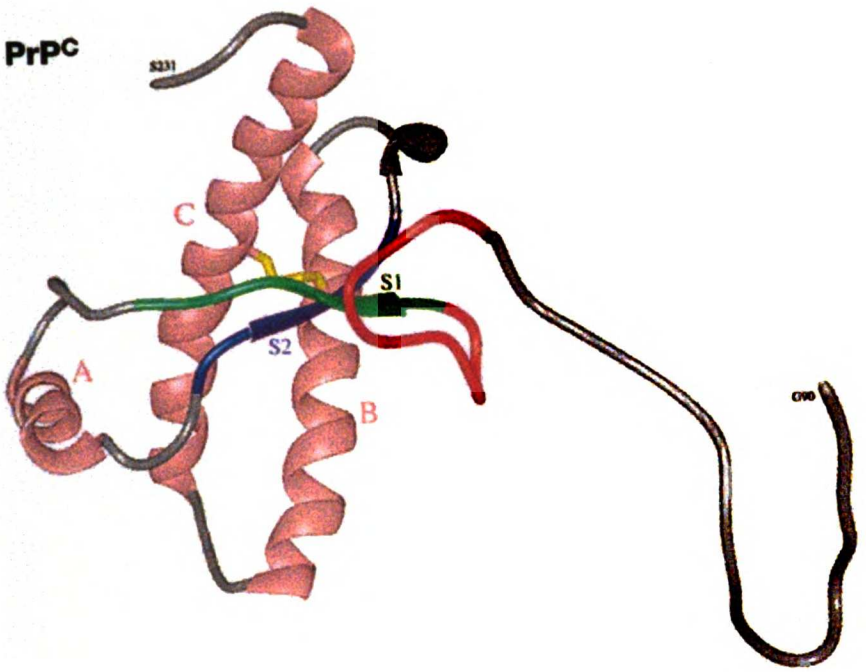
(Ch[Ca])(139) with associated differences in phenotype. D, Thermal denaturation of Ch fibers seeded by either Sc or Ca fibers (140).

Figure 8 Models depicting the relationship between transmission specificity and conformation. A, Robust species barrier between two variants of prion protein that do not form compatible conformations and thus do not cross-seed. B, A single polypeptide which can adopt two distinct conformations that allow assembly onto two otherwise incompatible prions.

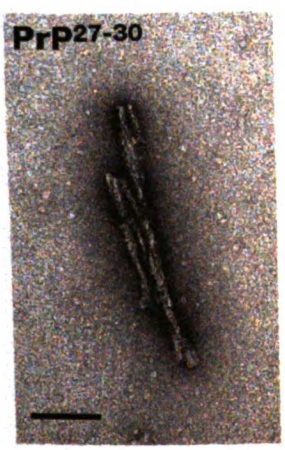
Figure 9 Two models for strain switching upon passage through a species barrier. A, In strain conversion, heterologous protein adopts a new conformation upon incorporation into prion seeds. B, In strain selection, host protein selects a compatible conformation from a heterogeneous innoculum. Over multiple rounds of prion replication, the distribution of conformations changes.

Chapter 1.
Figure 1

A PrP^{C}



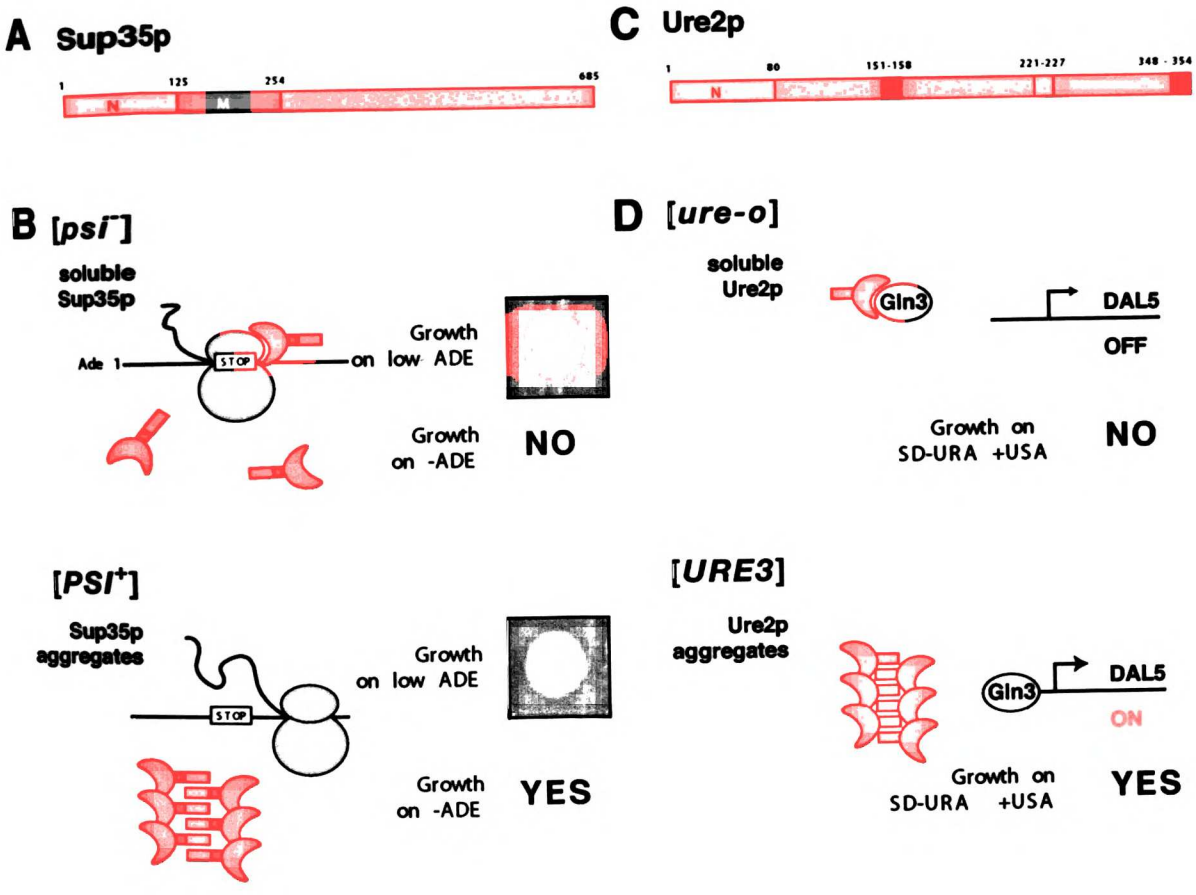
B PrP^{27-30}



C CONFORMATIONAL DIFFERENCES BETWEEN PrP^{C} and PrP^{SC}

PrP^{C}	PrP^{SC}
monomeric	multimeric
soluble	insoluble
protease sensitive	protease resistant
predominantly alpha-helical	predominantly beta-sheet

Chapter 1.
Figure 2



Chapter 1.
Figure 3

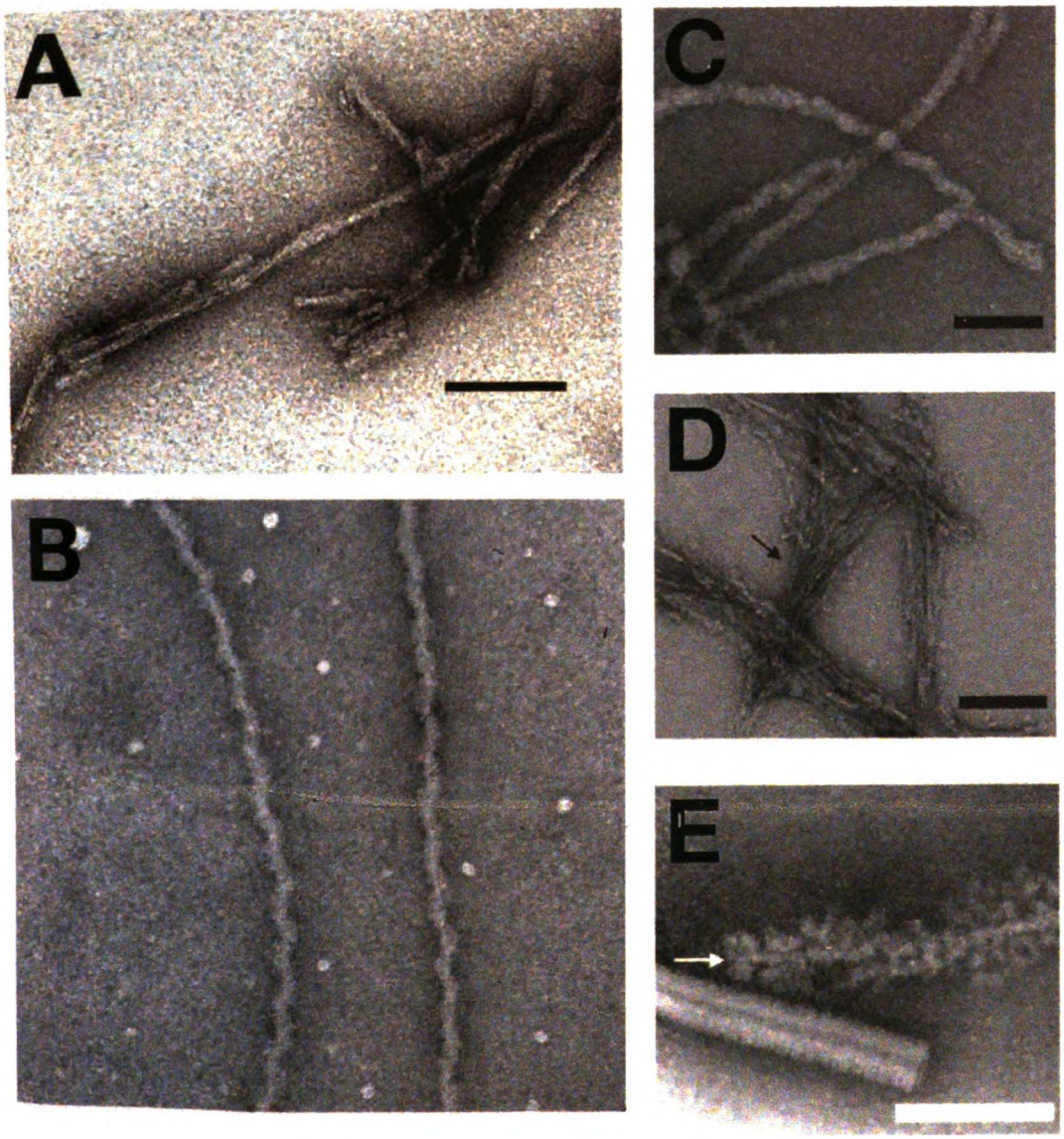


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Figure 4

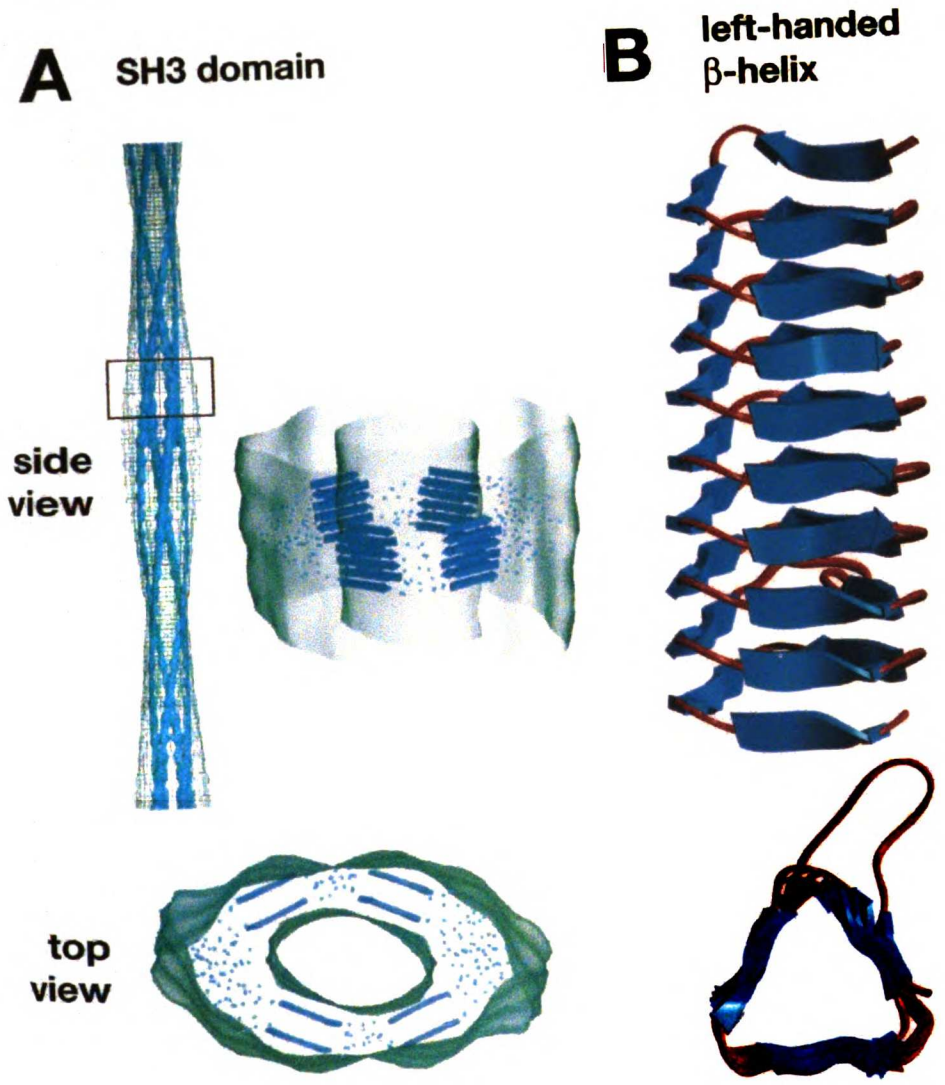

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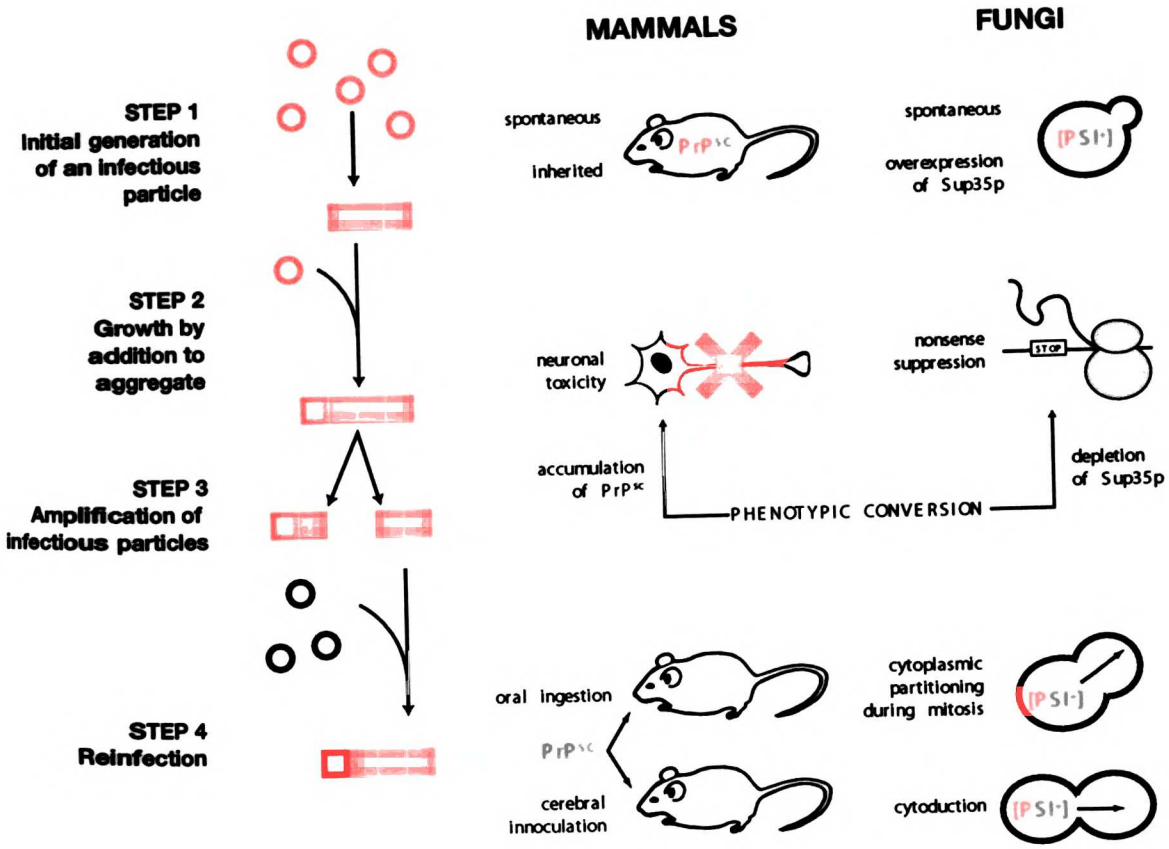
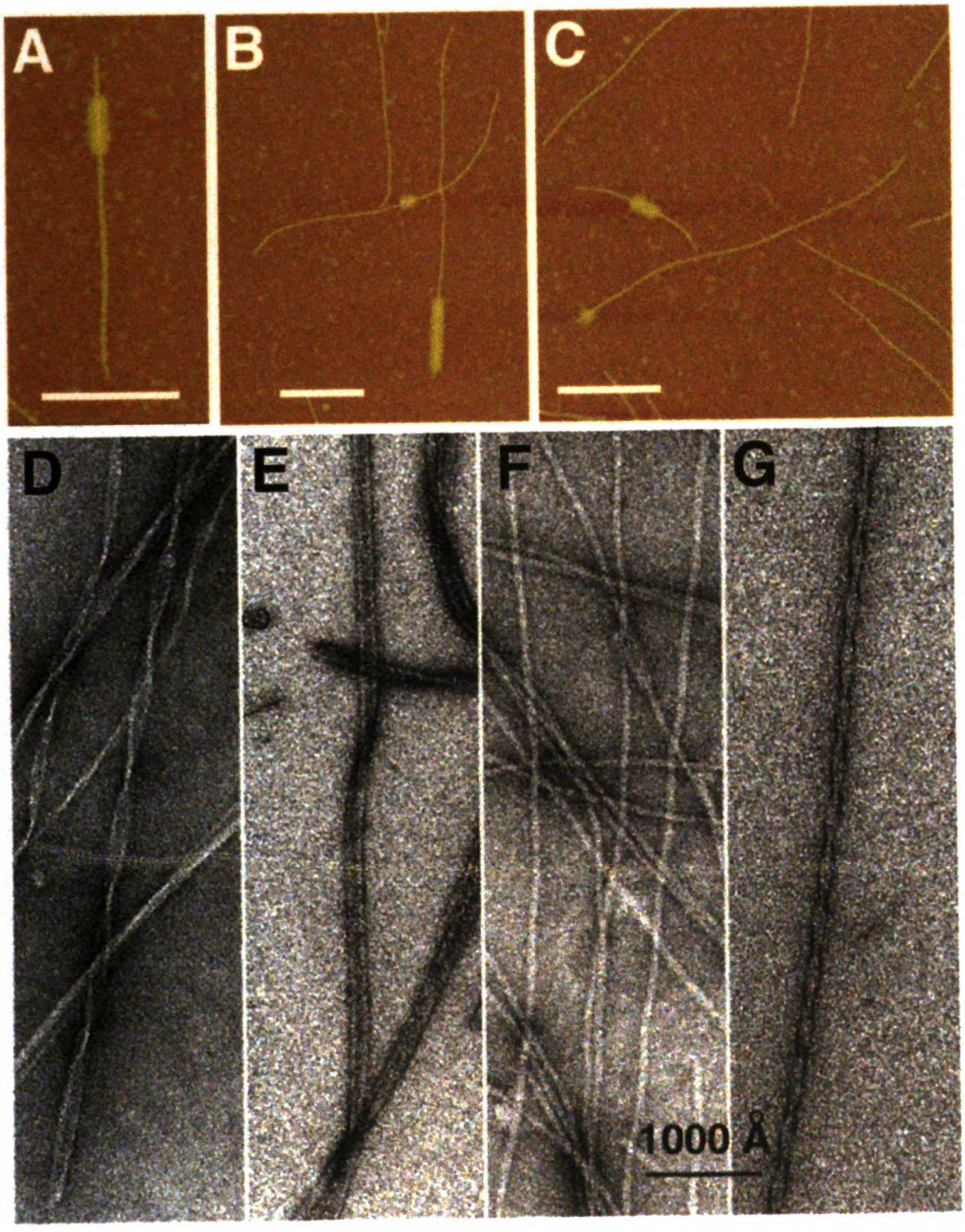
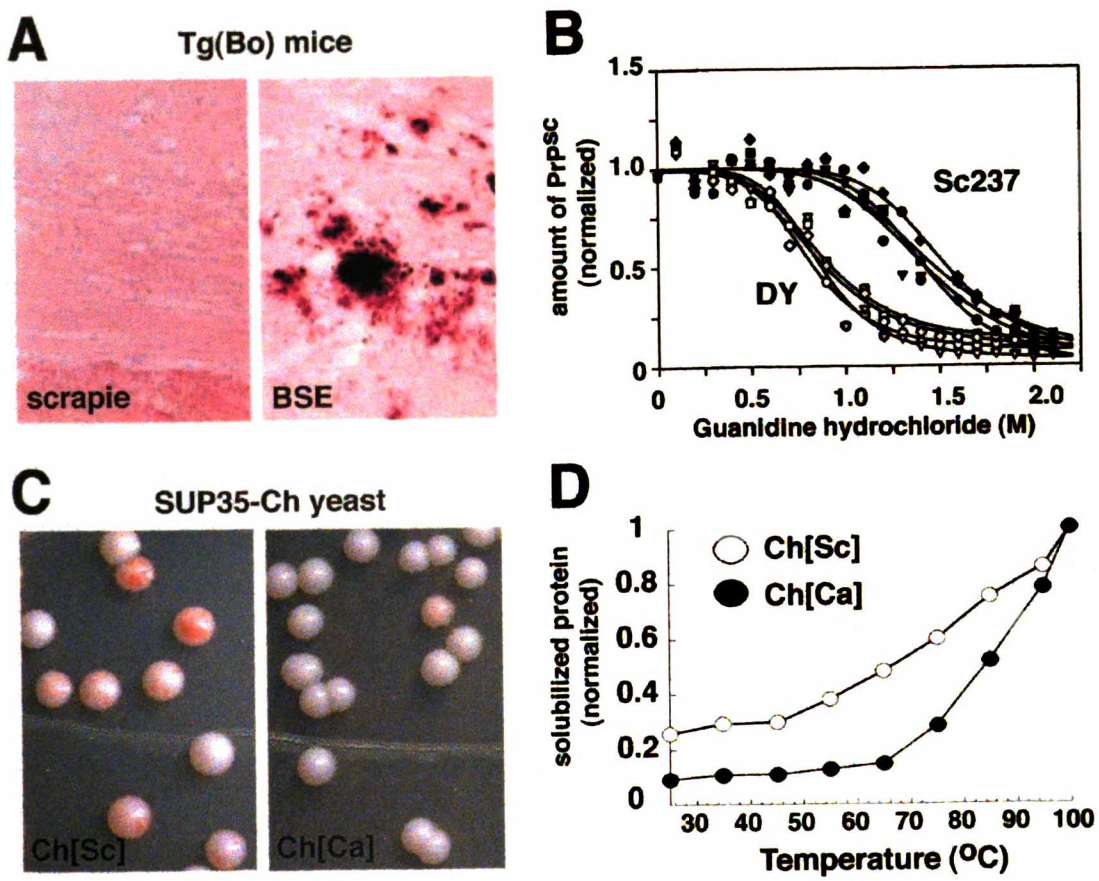
Figure 5



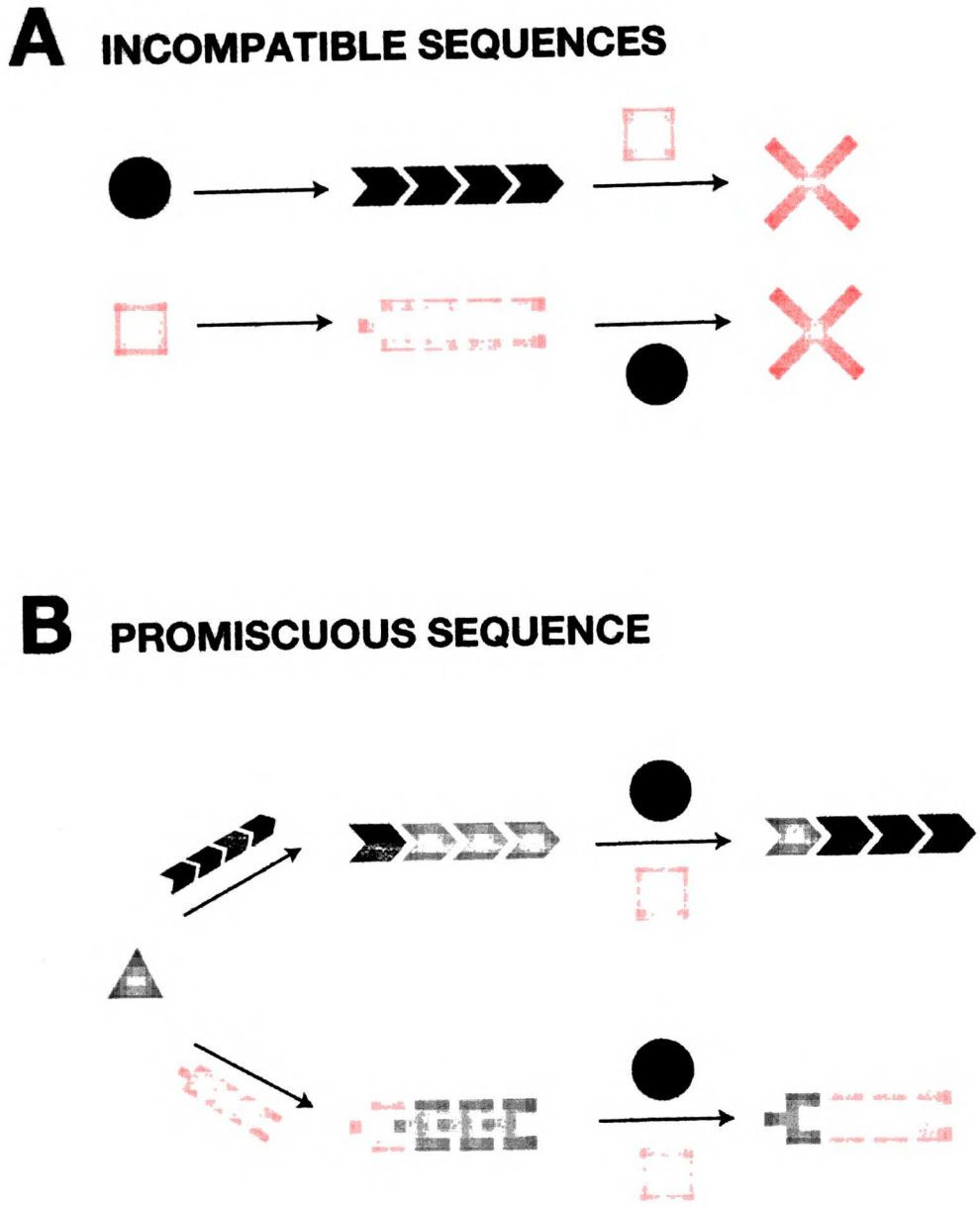
Chapter 1.
Figure 6



Chapter 1.
Figure 7

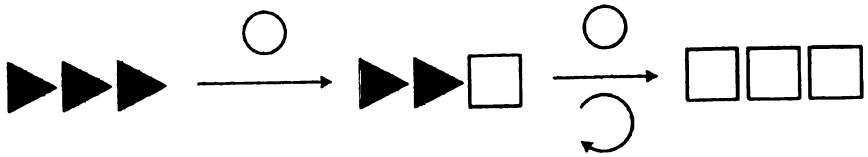


Chapter 1.
Figure 8

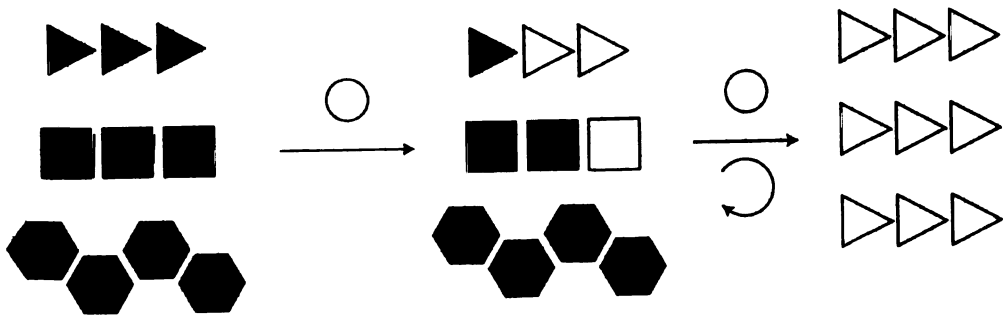


Chapter 1.
Figure 9

A **STRAIN CONVERSION**



B **STRAIN SELECTION**



Chapter 2

Cloning of SUP35 prion domains from a variety of yeast species.

My first project was under the guidance of Alex Santoso, to whom I am indebted for guiding my work. Alex's focus was on yeast prion species barriers that limit transmission between different species of prions. My contribution was the cloning of prion domains from a variety of budding yeast. I developed an adapter-mediated PCR strategy that took advantage of the highly conserved C-terminal domain of SUP35 in order to isolate and sequence SUP35 prion domains from *Candida albicans, Candida maltosa, Kluyveramyces lactis, Zygosacchromyces rouxi, Pichia methanolica* and *Pichia pastoris*. All homologs of SUP35 had functionally conserved prion domains in that they could form prions in a heterologous *S. cerevisiae* system, but were highly self-specific, indicative of a prion species barrier. These results are depicted in Figure 2. This figure and the experimental method below are part of a larger work published in the journal Cell in January 2000 under the title 'Molecular Basis of a Yeast Prion Species Barrier'.

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 reactions resulting in a single distinct band, PCR products were purified by agarose gel electrophoresis and sequenced. The Genbank accession numbers for Sup35p PrDM sequences are AF206287 (*C. albicans*), AF206288 (*K. lactis*), AF206289 (*K. marxianus*), AF206290 (*P. pastoris*), AF206291 (*S. ludwigii*), and AF206292 (*Z. rouxi*).

Oligonucleotide primers are as follows:

P10, AACGGTTGGGTCATCCATCTT;
P11, TTTGTTGGTATCCATGACCCATGACAAGTACCA;
P12:GGCCCAGTGAGCAGAGTGACGGAGGACTCGAGCTCAAGCTAATCCGGCGTGCAT
TGAC;
P13 GATCGTCAATGCACGCCGGATTTACGCC;
P14 CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
P15 ACCTGCCC;
Q0, CCAGTGAGCAGAGTGACG;
Q1, GACTCGAGCTCAAGCTAA;
AP-1, CCATCCTAATACGACTCACTATAGGGC;
AP-2, ACTCACTATAGGGCTCGAGCGGC.

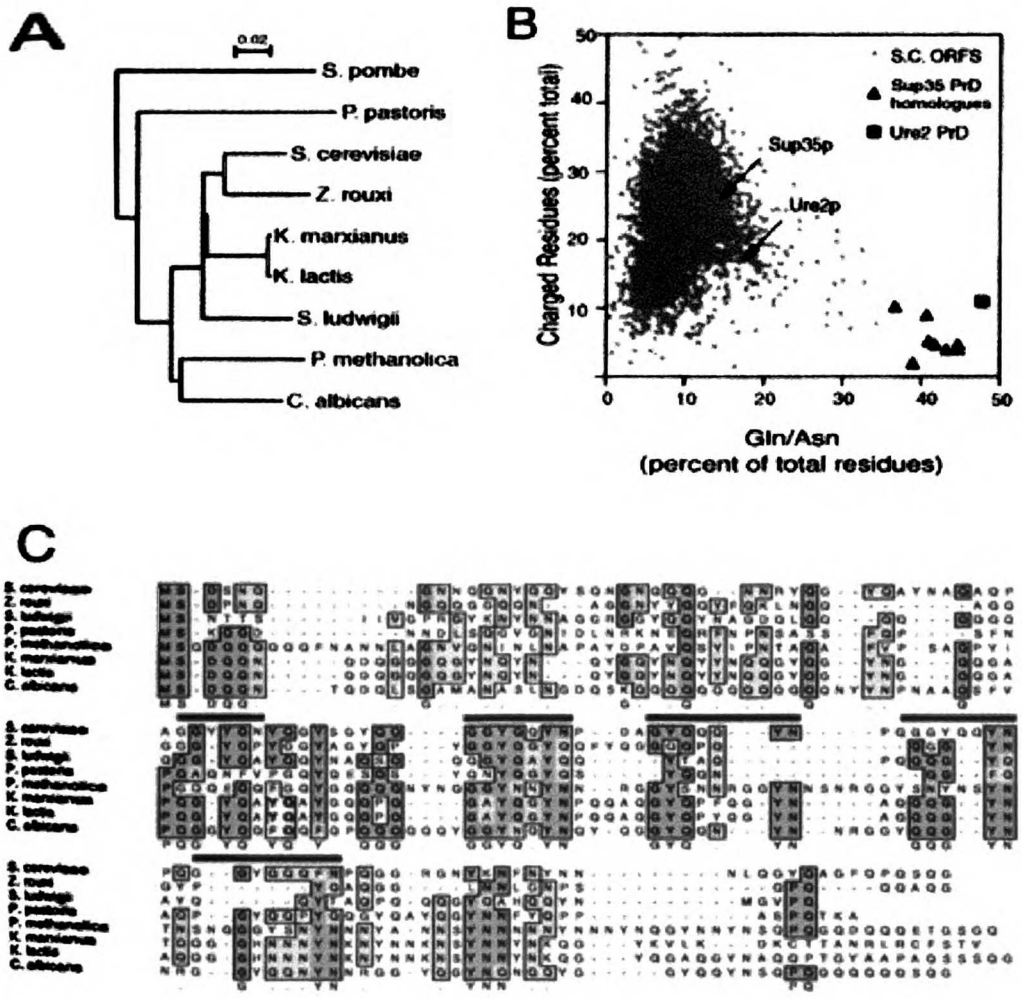
Figure 2 Evolutionary Analysis of Sup35p PrDs

(A) Phylogenetic relationship of yeast species based on 26S RNA sequences. For comparison, the fission yeast *S. pombe* whose Sup35p does not have a PrD, is shown. Scale (percent divergence) is denoted on top. Others 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. The black bar denotes the approximate location of the oligopeptide repeats.

Chapter 2
Figure 2



Chapter 3

Conformational diversity of yeast prions dictates seeding specificity.

This work, published in the journal Nature in March 2001, utilized a chimeric prion domain which could adopt two prion forms with distinct species specificity. This system, using both *in vitro* and *in vivo* experiments, showed that seeding-specificity could be encoded purely within the conformation of a protein aggregate.

Conformational diversity in a yeast prion dictates its seeding specificity

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A perplexing feature of prion-based inheritance is that prions composed of the same polypeptide can evoke different phenotypes (e.g., distribution of brain lesions), even when propagated in genetically identical hosts[1-3]. The molecular basis of this strain diversity and the relationship between strains and barriers limiting transmission between species remains unclear. Here we used the yeast prion phenomenon [*PSI*$^+$][4] to investigate these issues and examine the role conformational differences may play in prion strains[1,2,5]. We find that a chimeric fusion between the prion domains of two species (*Saccharomyces cerevisae* and *Candida albicans*) of Sup35p, the protein responsible for [*PSI*$^+$][4,6], forms alternate prion strains *in vivo* when initiated by transient overexpression of different Sup35p species. Similarly, purified chimera *in vitro,* when seeded with different species of Sup35p fibres, establishes and propagates distinct amyloid conformations. These fibre conformations dictate amyloid seeding specificity: chimera seeded by *S. cerevisiae* fibres efficiently catalyzes conversion of *S. cerevisiae* Sup35p but not *C. albicans* and vice versa. These and other considerations [1,2,5,7,8] argue that heritable prion strains result from self-propagating conformational differences within the prion protein itself. Moreover, these conformational differences appear to act in concert with the primary structure to determine a prion's propensity for transmission across a species barrier.

The [*PSI*$^+$] prion element of the yeast *S. cerevisiae* is transmitted through self-propagating aggregates of Sup35p, a component of the eukaryotic translation release factor[9]. Loss of soluble Sup35p in [*PSI*$^+$] cells leads to a heritable suppression of

nonsense mutations[4,6], which can be readily monitored using a nonsense mutation in the ADE1 gene (*ade1-14*)[10]. The suppression of *ade1-14* allows for growth on media lacking adenine and prevents the accumulation of metabolic intermediates that cause [*psi*$^-$] colonies to appear red on low adenine media. [*PSI*$^+$] propagation is mediated by the glutamine/asparagine rich amino terminal prion determining domain (PrD) [11,12]. Transient overexpression of this domain causes formation of *de novo* Sup35p prion aggregates *in vivo*[13] and purified PrD forms self-propagating amyloid fibres *in vitro*[14,15].

The prion forming ability of Sup35p is strongly conserved across budding yeast (Sacchromyceteles), but a barrier limits seeding between prion domains from different species[16-18]. For example, we previously observed that overexpression of *C. albicans* Sup35p PrD (CA) did not induce conversion of *S. cerevisiae* Sup35p to [*PSI*$^+$] and vice versa[17]. We found that a short region (e.g., residues 1-39) of *S. cerevisiae* PrD (SC) could confer susceptibility to seeding by *S. cerevisiae* prion when placed in *C. albicans* PrD. However, this region alone does not support prion formation[19] (unpublished observations). To further examine the relationship between primary structure and species specificity, we asked whether the extreme amino-terminus of CA was similarly required for susceptibility to *C. albicans* prion. These studies used a previously described chimeric PrD[17] (CHIM), composed of residues 1-39 of *S. cerevisiae* PrD and residues 40-140 from the *C. albicans* PrD. Specificity of prion formation was monitored using a set of isogenic *S. cerevisiae* yeast strains in which the sole chromosomal copy of *SUP35* consisted of SC, CA or CHIM PrDs fused to the carboxy-terminal translation termination domain (EF), designated SC-EF, CA-EF and CHIM-EF respectively. In these strains, the nonsense suppression phenotype depended on the state (aggregated or soluble) of the

relevant PrD fusions. To test for cross-species seeding, we used inducible plasmids containing the appropriate prion domain fused to GFP. Here, the nonsense suppression phenotype, following transient overexpression, reports on the ability of the "inducer" species to convert the chromosomal Sup35p.

Naively, if the extreme amino-terminus of CA were required for interaction with CA, we would have expected that CHIM would seed only itself and SC, but not CA. Instead, we found that CHIM was a promiscuous prion. Consistent with earlier results [17], overexpression of CHIM could induce formation of [*PSI*$^+$] in SC-EF (Fig. 1a), while CA overexpression did not; however, transient CHIM overexpression efficiently induced CA aggregation (Fig. 1b), despite the presence of the *S. cerevisiae*-derived extreme amino-terminus. Overexpression of SC, CA or CHIM also initiated a [*PSI*$^+$]-like state in a CHIM-EF background (Fig. 1c) which, like all characterized yeast prions, is readily cured by growth on low concentrations (5 mM) of guanidine hydrochloride (Fig. 1c inset) [17,20-22]. Thus, CHIM is itself a functional prion domain that bridges the species barrier between *C. albicans* and *S. cerevisiae.*

Although SC or CA overexpression induced prion formation of CHIM-EF with comparable efficiency, the resulting [*PSI*$^+$]-like phenotypes were dramatically different. Following selection of prion convertants on media lacking adenine, we examined their strain phenotypes by replating on low adenine media. This procedure revealed distinct colour phenotypes depending on the inducer species (Fig. 1d). Quantitative analysis (Fig. 1e) indicated that approximately 80% of the primary CHIM-EF prion inductants initiated by SC were either weak (pink) or unstable (sectored); in contrast, over 90% of CA

induced colonies had strong (white) phenotypes. Similar strain differences occur sporadically in new prion inductants of *S. cerevisiae*[3,23] and other species of Sup35p[16,17]. However, unlike previous work on spontaneously generated strains, an important advantage of the present system is that we can now control strain formation by changing the inducing species, thereby facilitating efforts to study this phenomenon *in vitro*.

We first established that purified CHIM could adopt an amyloid conformation by using a well-characterized *in vitro* system[14,15] assayed with the amyloid-specific dye Congo red[12]. Similar to wildtype SC polymerization, spontaneous conversion of CHIM was accompanied by a lag phase of about 50 minutes which was eliminated by the addition of a small amount (3% mol/mol) of the fibres formed at the reaction endpoint (Fig. 2a). As in previous studies [24], we found that the kinetics of Congo red binding mirrored formation of fibres observed by electron microscopy (Fig. 2b), increased binding to thioflavin-T and resistance to solubilization by SDS in the absence of boiling (data not shown). The spontaneously formed CHIM fibres readily seeded SC (Fig. 2c); however, contrasting our *in vivo* results, little seeding of CA polymerization was observed (Fig. 2d). No such difference between *in vivo* and *in vitro* seeding had been previously reported in over a dozen experiments with different species and mutants of Sup35p, [12,17,19,25,26], a point which we address below.

Next we established that induction of CHIM by SC or CA could lead to different fibre conformations. Akin to *in vivo* results, both SC fibres and CA fibres efficiently seeded conversion of CHIM protein (Fig. 3a). Polymerization of CHIM monomer initiated by SC fibres (CHIM[SC]) led to a monotonic increase in apparent Congo red

binding; however, CHIM monomer seeded by CA fibres (CHIM[CA]) showed a fast rise and steady decline in apparent Congo red binding. This profile was highly reminiscent of CA polymerization seeded by CA (Fig. 3a inset). It is important to note that the loss of apparent Congo red binding is not due to depolymerization since electron microscopy and thioflavin-T binding confirmed the persistence of amyloid (data not shown). Moreover, brief sonication of the final material (data not shown) or increasing shear during conversion, as is done in subsequent CA experiments (Fig. 4b), restored apparent Congo red binding. Hence, the decrease in dye binding results from a prion conformation characteristic of CA and CHIM[CA] fibres.

The two distinct CHIM fibre conformations propagated stably (Fig. 3b) as the characteristic differences in apparent Congo red binding were also observed in second generation fibres in which CHIM was seeded by CHIM[SC] or CHIM[CA], denoted CHIM[[SC]] and CHIM[[CA]] respectively. Limited proteolysis and detection by Western blot analysis [24] confirmed the existence of conformational differences between the fibres (Fig. 3c). Chymotrypsin patterns obtained from CHIM[CA] and CHIM[[CA]] were substantially different from those derived with CHIM[SC] and CHIM[[SC]]. These differences were retained throughout the timecourse of digestion (data not shown), arguing that these patterns reflected real conformational differences rather than differences in protease sensitivity. We conclude that distinct fibre conformations could be propagated with pure recombinant proteins.

We next examined the effect of these conformational differences on CHIM's seeding specificity. Here we used second generation fibres, which contain only 0.075%

residual parent seed, an amount insufficient to affect conversion of either SC or CA polymerization (data not shown). As with spontaneously converted CHIM fibres, CHIM[[SC]] seeded SC monomer (Fig. 4a) whereas CHIM[[CA]] minimally affected the lag phase of SC polymerization. Remarkably, CHIM[[CA]] gained the ability to seed CA monomer while addition of either CHIM[[SC]] (Fig. 4b) or spontaneously polymerized CHIM (Fig 2d.) only modestly affected CA polymerization. We observed similar specificity when we monitored polymerization by thioflavin-T binding (data not shown) or formation of SDS-resistant aggregates (Fig. 4c). Therefore, the chimera is capable of adopting two distinct amyloid fibre conformations with different species preferences. The existence of two discernable fibre conformations suggests an explanation for why spontaneously formed CHIM fibres failed to seed CA *in vitro* (Fig. 1b): unseeded CHIM fibres have a preference for the SC-specific conformation. Consistent with this, the proteolytic pattern (Fig. 3c) and Congo red binding properties (Fig. 2a) of spontaneously polymerized CHIM resembled that of CHIM[SC] and CHIM[[SC]], arguing that they share a common conformation.

Originally attributed to differences in the nucleic acid sequence of a hypothetical slow virus, strains now appear to be an intrinsic feature of conformation-based inheritance in both mammalian[1,2] and yeast prions[3,16,17,23]. Two questions arise: What are the molecular requirements for establishing and maintaining strains? What is the relationship between strains and cross-species transmission? Here we establish that a pure protein, devoid of covalent modifications[7] and contaminants such as lipids or polysaccharides, which could act as scaffolds[27,28], can adopt conformationally distinct fibres. These conformations are initially determined by the parent seed, but afterwards

propagate stably. In the mammalian prion system, it has been shown that strains are accompanied by differences in prion conformation[1,5,8] and these differences can be propagated in a partially purified *in vitro* system[2,29]. Taken together, these observations strongly support the idea that a single protein can adopt a variety of different self-propagating prion conformations and that these conformational differences are the basis for the diversity of strain phenotypes.

Our results also establish a link between a prion's conformation (and by inference the strain associated with it) and seeding specificity. The CHIM strain seeded by SC efficiently initiates polymerization of SC but has only modest effect on CA conversion, while CA induced CHIM adopts a strain that resembles CA and specifically catalyzes polymerization of CA. These data suggest that a major part of a robust species barrier, like that between *S. cerevisiae* and *C. albicans* (Fig. 4d), is due to the propensities of the two sequences for formation of distinct non-interacting conformations. However, a promiscuous prion (in our case, CHIM) can access multiple conformations, yet once templated, faithfully propagates the specificity and conformation of the initial seed. Thus, species barriers and strains are intimately related phenomena: a prion's primary structure dictates the spectrum of favoured strain conformations, whereas a given prion strain infects only species capable of adopting prion conformations compatible with that strain. The ability of strains to modulate prion specificity could help explain the apparent transmission of bovine spongiform encephalopathy (BSE or mad cow disease) to a variety of mammals, including human beings[7,8]. On a more speculative note, it is possible that the process of infection, rendering, and reinfection, which led to the BSE

epidemic, also resulted in the selection and specific amplification of a prion strain with enhanced virulence.[30]

Methods

Plasmid construction. URA marked inducer plasmids containing GFP fusions of SC, CA or CHIM (426CpSCGFP, 426CpCAGFP, 426CpSC(1-39)CAGFP respectively) were generated in a previous study[17] using the copper inducible CUP1 promoter for overexpression experiments. Integrative vectors were generated via cloning of the relevant prion forming domains from these plasmids into URA marked integration plasmids together with the native promoter region of *SUP35*, a highly charged middle domain and the carboxy-terminal translation termination domain of native Sup35p.

Gene integration and replacement. Integrative plasmids were linearized via restriction enzyme digest (MscI) within the native promoter region and transformed into *S. cerevisiae* yeast strain 74D-94 [*PSI*$^+$] and [*psi*$^-$][10]. Replacement of native allele was done through gamma integration and passage on media containing 5-fluoro-orotic acid to select for recombination excision events. The presence of the expected prion determining domain (either CA or CHIM) was detected by PCR and Western blot analysis confirmed proper expression of integrated domains.

Induction of prion states. Induction experiments were performed as before[17]. Transformants with SC, CA or CHIM inducer plasmids were grown in SD-Ura media and induced with 50 μM $CuSO_4$ for 24 hours. Saturated cultures were plated on SD-Ade media and visible colonies were counted after five days growth at 30°C. To determine strain phenotype, inductants from SD-Ade plates were grown overnight in liquid SD-Ade

media and plated on non-selective low Ade media. Colonies were counted and binned after three days growth.

Conversion to amyloid. 9x (CA and CHIM) or 7x (SC) his-tagged protein was purified in denaturing conditions as previously described[17]. Experiments with non-tagged protein showed similar results as with tagged protein (unpublished observations). Polymerization and Congo red binding assays were performed as before[17]. All seeding reactions used 3% mol/mol of sonicated fibres. In CA experiments, with the exception of the inset of Fig. 3a, a small glass bead (3 mm in diameter) was added, which inhibits loss of Congo red binding, perhaps by increasing shear, while only modestly altering polymerization kinetics. SDS solubility was assayed as previously published[24] with Coomassie staining for protein detection. Electron microscopy was done as before[12].

Limited proteolysis and western analysis. 10 μM fibres in 150 mM NaCl, 5 mM KH_2PO_4, pH 7.4, were incubated with chymotrypsin (1/125 mol/mol) for 15 minutes at room temperature. Reactions were quenched by addition of SDS-PAGE loading buffer and incubation at 100 °C for 10 minutes. Digests were run on 16% Tris/Tricine gels (Novex) and transferred to nitrocellulose. Presence of protein was detected by immunoblotting with polyclonal antibodies to *S. cerevisiae* Sup35 prion determining domain and detected by chemiluminescence (SuperSignal West Pico materials; Pierce).

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

2. Bessen, R. A. *et al.* Non-genetic propagation of strain-specific properties of scrapie prion protein. *Nature* **375**, 698-700 (1995).

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

4. Serio, T. R. & Lindquist, S. L. [PSI+]: an epigenetic modulator of translation termination efficiency. *Annu Rev Cell Dev Biol* **15**, 661-703 (1999).

5. Telling, G. C. *et al.* Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science* **274**, 2079-2082 (1996).

6. Paushkin, S. V., Kushnirov, V. V., Smirnov, V. N. & Ter-Avanesyan, M. D. Propagation of the yeast prion-like [psi+] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor. *EMBO J* **15**, 3127-3134 (1996).

7. Collinge, J., Sidle, K. C., Meads, J., Ironside, J. & Hill, A. F. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature* **383**, 685-690 (1996).

8. Collinge, J. Variant Creutzfeldt-Jakob disease. *Lancet* **354**, 317-323 (1999).

9. Stansfield, I. *et al.* The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in Saccharomyces cerevisiae. *EMBO J* **14**, 4365-4373 (1995).

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

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

12. DePace, A. H., Santoso, A., Hillner, P. & Weissman, J. S. A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion. *Cell* **93**, 1241-1252 (1998).

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

14. King, C. Y. *et al.* Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. *Proc Natl Acad Sci U S A* **94**, 6618-6622 (1997).

15. Glover, J. R. *et al.* Self-seeded fibers formed by Sup35, the protein determinant of [PSI+], a heritable prion-like factor of S. cerevisiae. *Cell* **89**, 811-819 (1997).

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

17. Santoso, A., Chien, P., Osherovich, L. Z. & Weissman, J. S. Molecular basis of a yeast prion species barrier. *Cell* **100**, 277-288 (2000).

18. Chernoff, Y. O. *et al.* Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein. *Mol Microbiol* **35**, 865-876 (2000).

19. Liu, J. J. & Lindquist, S. Oligopeptide-repeat expansions modulate 'protein-only' inheritance in yeast. *Nature* **400**, 573-576 (1999).

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

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

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

23. Zhou, P. *et al.* The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. *EMBO J* **18**, 1182-1191 (1999).

24. Serio, T. R. *et al.* Nucleated conformational conversion and the replication of conformational information by a prion determinant. *Science* **289**, 1317-1321 (2000).

25. Paushkin, S. V., Kushnirov, V. V., Smirnov, V. N. & Ter-Avanesyan, M. D. In vitro propagation of the prion-like state of yeast Sup35 protein. *Science* **277**, 381-383 (1997).

26. Kochneva-Pervukhova, N. V. *et al.* Mechanism of inhibition of Psi+ prion determinant propagation by a mutation of the N-terminus of the yeast Sup35 protein. *EMBO J* **17**, 5805-5810 (1998).

27. Appel, T. R., Dumpitak, C., Matthiesen, U. & Riesner, D. Prion rods contain an inert polysaccharide scaffold. *Biol Chem* **380**, 1295-1306 (1999).

28. Klein, T. R., Kirsch, D., Kaufmann, R. & Riesner, D. Prion rods contain small amounts of two host sphingolipids as revealed by thin-layer chromatography and mass spectrometry. *Biol Chem* **379**, 655-666 (1998).

29. Kocisko, D. A. *et al.* Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. *Proc Natl Acad Sci U S A* **92**, 3923-3927 (1995).

30. Bartz, J. C., Bessen, R. A., McKenzie, D., Marsh, R. F. & Aiken, J. M. Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. *J Virol* **74**, 5542-5547 (2000).

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Figure 1. Characterization of CHIM prion formation *in vivo*. **a-c**, Induction experiments carried out in an SC-EF (**a**), CA-EF (**b**) or CHIM-EF (**c**) background, using the indicated species of inducer prion. Percentages of Ade^+ colonies are shown. Control denotes empty vector. Increased induction of SC-EF by CHIM compared to SC is most likely due to a higher rate of *de novo* aggregation of the CHIM prion domain, as observed by fluorescence microscopy (unpublished observations) and spontaneous prion formation (**c**). inset: Induced chimera prion [X^+] shows curing after passage on 5 mM guanidine HCl containing media [X^+(Gdn)], also shown are [*PSI*$^+$] and [*psi*$^-$] yeast for comparison. **d**, Examples of phenotypes of independently induced prion states of CHIM-EF with SC (upper) or CA (lower) inducers. CHIM-EF was induced by transient overexpression of the indicated inducer Sup35p species and prion convertants were selected on minus Ade media. For each inducer species, we pooled multiple convertants, back diluted and plated on low Ade media to reveal strain phenotypes. **e**, Quantitative analysis of CHIM strains generated in **c**. Colonies were grouped according to colour (white, pink, sectored). A representative picture of each colony type is shown above the appropriate bin.

Figure 2. CHIM forms self-seeded amyloid fibres *in vitro*. **a**, Congo red binding assays show that purified CHIM monomer spontaneously forms amyloid with a lag phase (diamonds) which is abolished upon addition of 3% (mol/mol) preformed fibres (circles). Abscissa represents moles of Congo red bound per mole protein. **b**, Electron microscopy indicates that CHIM forms typical amyloid

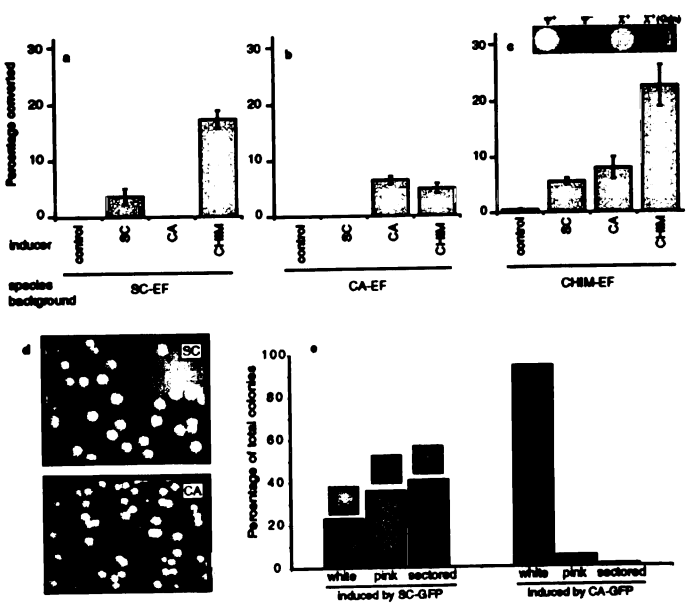
fibres. Bar denotes 100 nm. **c**, CHIM fibres from **a** catalyze SC polymerization (circles) compared to unseeded reactions (diamonds). **d**, Same CHIM fibres have only a modest effect on CA polymerization (circles) compared to unseeded CA reactions (diamonds).

Figure 3. Generation and propagation of distinct CHIM fibre conformations. **a**, Polymerization of CHIM in the absence (diamonds) or presence of 3% SC fibres (circles) or CA fibres (triangles). n.b. slow decrease in apparent Congo red binding for CA initiated CHIM polymerization mirrors CA self-seeded polymerization (inset). **b**, Effect of CHIM[SC] (circles) and CHIM[CA] (triangles) on CHIM polymerization. Schematic representation of repeated seeding for demonstration of conformation propagation shown at top. **c**, Western blot analysis of first ([SC] and [CA]) and second ([[SC]] and [[CA]]) generation CHIM fibres incubated with (+) or without (-) chymotrypsin. Also shown is the digest of spontaneously polymerized CHIM fibres (SPONT). Relative molecular masses (in kiloDaltons) are indicated.

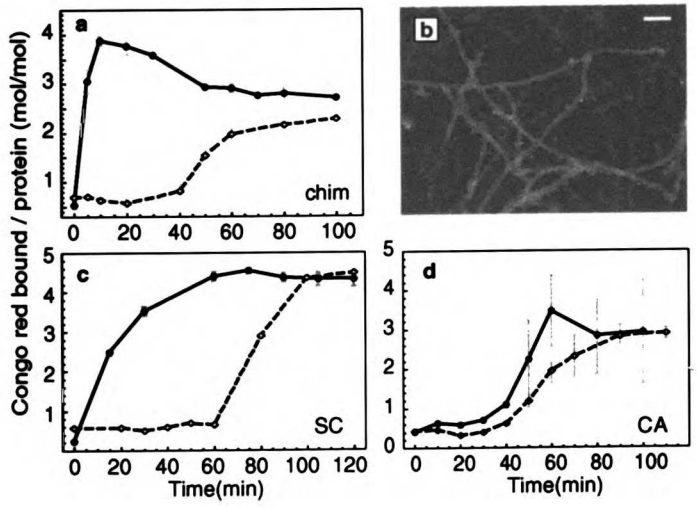
Figure 4. CHIM amyloids retain specificity of parent seed. **a**,**b** Second generation CHIM fibres ([[SC]] or [[CA]]) were used to seed either SC (**a**) or CA (**b**) polymerization. In the case of CA polymerization, a small glass bead was added to inhibit loss of apparent Congo red binding (see methods). **c**, SDS-PAGE analysis of polymerization reactions (SC or CA monomer), seeded with the indicated fibre. Aliquots taken at 30 minutes, before spontaneous polymerization begins, but after seeded polymerization was largely completed.

Appearance of band after boiling (lower) shows that loss of band in absence of boiling (upper) is due to polymerization, not loss of protein. **d**, Model depicting relationship between species barrier and prion conformation. Sequences with different strain conformation propensities (SC or CA) have a robust transmission barrier (top). A promiscuous prion (CHIM) can adopt either conformation (bottom), but once incorporated, retains the strain conformation and seeding specificity of the initial seed.

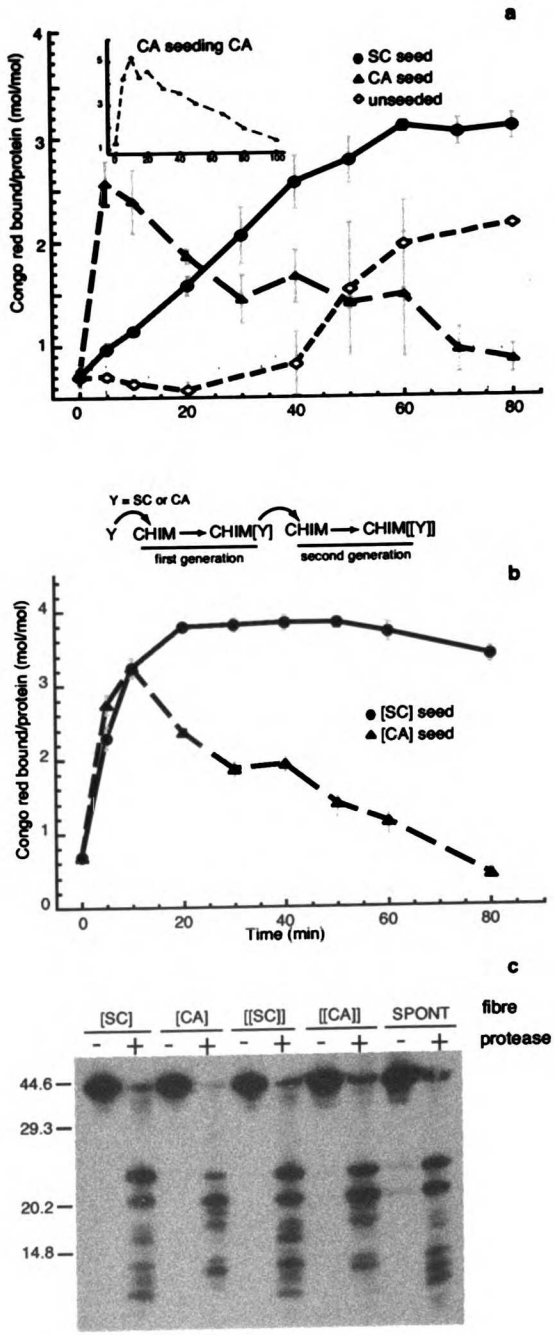
Chapter 3
Figure 1



Chapter 3
Figure 2



Chapter 3
Figure 3



Chapter 3
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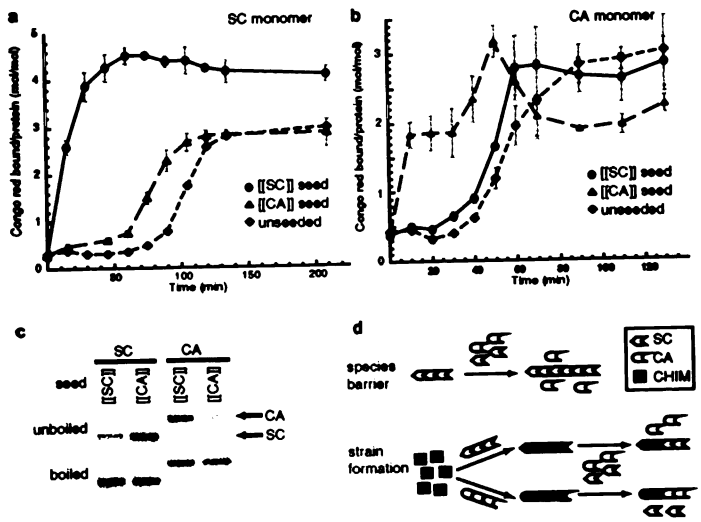

Chapter 4

Generation of a yeast prion species barrier by mutational control of amyloid conformations.

This work demonstrated that a species-specific yeast prion transmission barrier can be generated by manipulating the spectrum of conformations adopted by a yeast prion. This could be done *in vivo* and *in vitro* by using point mutations that favored one conformation over another and also by changing environmental conditions to favor particular conformations. This body of work was published in the journal Nature in October 2003.

Generation of prion transmission barriers by mutational control of amyloid conformations

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Self-propagating beta-sheet rich protein aggregates are implicated in a wide range of protein misfolding phenomena, including amyloid diseases and prion-based inheritance[1]. Two properties have emerged as common features of amyloids. Amyloid formation is ubiquitous: many unrelated proteins form such aggregates and even a single polypeptide can misfold into multiple forms[2-6], a process thought to underlie prion strain variation[7]. Despite this promiscuity, amyloid propagation can be highly sequence specific: amyloid fibres often fail to catalyze aggregation of other amyloidogenic proteins[8,9]. In prions, this specificity leads to barriers limiting transmission between different species[7,8,10-12]. Using the yeast prion [*PSI*$^+$][13], we show *in vitro* that point mutations in Sup35p, the protein determinant of [*PSI*$^+$], alter the range of 'infectious' conformations, which in turn changes amyloid seeding specificity. We generate a new transmission barrier *in vivo* by using these mutations to specifically disfavour subsets of prion strains. The ability of mutations to alter the conformations of amyloid states without preventing amyloid formation altogether provides a general mechanism for the generation of prion transmission barriers and may help explain how mutations alter toxicity in conformational diseases.

The yeast prion [*PSI*$^+$][13] is a non-Mendelian element caused by self-propagating aggregates of the translation termination factor Sup35p, which leads to a nonsense suppression phenotype. In strains containing an *ade1* gene with a nonsense mutation, [*PSI*$^+$] colonies are white and grow on media lacking adenine, while [*psi*$^-$] colonies are red and require adenine. [*PSI*$^+$] can be induced *de novo* by transient overexpression of the modular amino-terminal glutamine/asparagine-rich prion domain, which is necessary for prion propagation and forms self-seeding amyloid fibres *in vitro*[4,14]. While the prion domain of Sup35 is conserved across a range of

budding yeast, [*PSI*$^+$] 'infectivity' is often limited by transmission barriers[8,10-12] analogous to the species barriers seen in mammalian prions. For example, prion domains of Sup35p from *S. cerevisiae* (Sc) and *C. albicans* (Ca), while independently capable of forming heritable conformations, do not cross-seed each other *in vivo* or *in vitro*[8]. (n.b, prion domains are referred to by two letter species abbreviations and mutations where appropriate, Table S1). [*PSI*$^+$], like mammalian prions and other yeast prions, exhibits a range of heritable phenotypic strain variants[15], which are linked to differences in the conformation of the infectious prion protein although cellular factors might also contribute to their propagation[7,13]. [*PSI*$^+$] strain variants differ in mitotic stability[15], in interactions with the cellular chaperone machinery [16], and in solubility and activity of Sup35p protein[15,17-19]. Sup35p aggregates purified from different [*PSI*$^+$] strains also differ in their ability to seed purified Sc polymerization *in vitro*[19]. In addition, strains can play a major role in determining specificity of prion transmission: [*PSI*$^+$] prion strains differ greatly in their ability to recruit Sup35 mutants[20]. Similarly, a chimeric prion (Ch), comprising the first forty amino acids of Sc fused to the remainder of the Ca prion domain, is able to form at least two amyloid conformations, one that seeds Sc (termed Ch[Sc]) and another that seeds Ca (Ch[Ca])[21]. (n.b., different amyloid conformations are referred to by the fibre protein followed by seed used to initiate polymerization in brackets, Table S1.) This link between prion strains and transmission barriers is likely to be general as the mammalian species barrier is also strain dependent[7].

While Ch polymerization induced by an Sc or Ca template robustly produces the Ch[Sc] or Ch[Ca] conformations[21] (Fig. 1), we show here that conformations of Ch fibres formed in absence of seed are highly sensitive to the polymerization conditions. As assessed by both seeding specificity (Fig. 1a, b) and by more direct

physical measurements such as protease protection (Fig. S1) or resistance to denaturation (Fig 1c), polymerization of Ch at 15 C shows a strong bias for forming the Ch[Sc] conformation, while Ch seeds formed at 37 C adopt a Ch[Ca] conformation. Polymerization at intermediate temperatures yields a mixture of both conformations (see below). Once formed, the two Ch conformations are highly stable and propagate robustly, e.g, Ch polymerized at 15 C with seeds formed at 37 C retain the seeding specificity of Ch polymerized at 37 C (Fig. S2).

If, as suggested by the above data, the choice among different prion forms is under kinetic control then mutations which differentially affect the rate of formation of individual amyloid conformations should shift the spectrum of self-propagating prion states. In the case of the Ch protein, where the Ch[Sc] and Ch[Ca] prion forms have different seeding specificity, mutations that selectively slow one pathway should generate a transmission barrier (Fig. 2a). We tested this by examining whether a series of point mutations known to slow Sc prion formation[22] would also specifically inhibit the Ch[Sc] pathway. We grafted two of these mutations onto the Sc-derived amino-terminus of the Ch prion: Q15R, which moderately disables Sc prion formation (yielding ChQ15R), and S17R, which has a stronger effect[20,22] (yielding ChS17R).

As predicted, these mutations create a transmission barrier against wildtype Sc protein *in vitro* by disfavouring the Ch[Sc] conformation. While ChQ15R retained the ability to be seeded by Sc or Ca fibres, ChS17R specifically lost the ability to be seeded by Sc (Fig. 2b). Furthermore, under conditions (25 C) where Ch adopts a mixture of conformations capable of seeding Sc or Ca, ChQ15R, and to a greater extent, ChS17R, form seeds that show a strong preference for seeding Ca (Fig. 2c). In principle this change in specificity could be due to the mutations *per se*, rather than a

change in fibre conformation. We exclude this possibility by showing that fibres formed by seeding Ch with spontaneously polymerized ChQ15R or ChS17R mutants adopted a Ch[Ca] state (Fig. 2d). Moreover, ChQ15R can be forced into a Ch[Sc]-like form when templated by Sc fibres, as evidenced by the seeding specificity and stability of ChQ15R[Sc] (Fig. S3).

The hypothesis that differences in prion strains result from different conformations of the prion protein suggests that an *in vitro* shift in Ch mutant forms should be accompanied by a similar shift in prion strain phenotypes *in vivo*. To test this, we examined the spectrum of prion strains induced by transient overexpression of the various prion domains using yeast (*Saccharomyces cerevisiae*) in which the genomic *SUP35* prion domain was replaced by the Ch sequence (*SUP35*-Ch yeast). (n.b., yeast strains are denoted by the species of *SUP35* prion domain encoded by the genomic *SUP35* gene, Table S1). As previously reported, using an Sc inducer favours formation of 'weak' Ch prion states (determined by colony colour on low ADE media as a readout of *ade1* nonsense suppression readthrough) while Ca overexpression leads to a preponderance (~90 percent) of strong variants[21](Fig. 3a). Induction with Ch resulted in a strain distribution falling between those seen with the Sc or Ca inducers whereas the ChQ15R and ChS17R mutants show a progressive shift towards strong "Ch[Ca]-like" strains (Fig. 3a). These data argue that *in vivo* Ch also spontaneously forms both Ch[Sc] and Ch[Ca] conformations and that the Q15R and S17R mutations specifically disfavour formation of weak [*PSI*$^+$] which we hypothesize are in the Ch[Sc] state.

This shift in strain preference was accompanied by creation of a transmission barrier *in vivo*. While *SUP35*-Ch yeast are promiscuous, capable of being converted

to the prion state by either Sc or Ca overexpression, *SUP35*-ChQ15R yeast, and to a greater extent *SUP35*-ChS17R yeast, specifically lost susceptibility to induction by Sc (Fig. 3b). This transmission barrier was reciprocal as overexpression of ChQ15R or ChS17R could efficiently induce the prion state in *SUP35*-Ca yeast, but not in *SUP35*-Sc yeast (Fig. 3c).

Taking advantage of a set of four glycine to alanine mutations that slowed polymerization of Ca (P.C. and J.S.W., unpublished data), we produced a variant of Ch (ChGA4, Fig. S4) with a decreased propensity to form the Ch[Ca] state. *In vivo*, ChGA4 overexpression efficiently induces prion formation in *SUP35*-Sc yeast, but not in *SUP35*-Ca yeast - a specificity reciprocal to that seen with ChS17R (Fig. 3d). Similarly, *in vitro,* ChGA4 spontaneously forms amyloid fibres that preferentially seed Sc over Ca. This specificity appears to result from a change in fibre conformation, rather than by a direct effect of the mutations, as fibres generated by seeding Ch with spontaneously polymerized ChGA4 adopted a Ch[Sc] state (Fig. 3e). Taken together these data argue that the Ch mutations generate transmission barriers both *in vivo* and *in vitro* by specifically disfavouring subsets of Ch amyloid conformations.

We next examined whether point mutations could alter prion conformations/strains adopted by the natural *S. cerevisiae* Sup35 prion domain (Sc), which, like many amyloid-prone proteins[5] including another yeast prion protein Ure2p[3], is known to form a range of self-propagating states[2,4]. While transient overexpression of wildtype or the ScQ15R derivative induced a similar spectrum of strong (white or lightly sectored) [*PSI*$^+$] variants in a *SUP35*-Sc background (74-D694), induction by the ScS17R mutant strongly favoured formation of weak strains

(mostly red or heavily sectored) (Fig. 4a). *In vitro*, we examined the effects of Q15R and S17R mutations on Sc amyloid conformations using a recently described single fibre assay based on atomic force microscopy that follows the growth of unlabelled monomers as they add onto the ends of antibody-labeled seeds[2]. Previously, this assay demonstrated that Sc spontaneously formed several distinct fibre types that differ both in polarity and overall rate of growth (Fig. 4b). The range of these fibre types is highly dependent on sequence, as revealed by a marked difference in growth patterns seen between self-seeded Sc, ScQ15R, or ScS17R reactions (Fig. 4b). These changes stem from propagating conformational differences within the fibres, as addition of wildtype Sc monomer onto either ScQ15R or ScS17R seeds resulted in single-fibre growth patterns distinct from the pattern of multiple peaks seen when wildtype adds onto wildtype seeds. Interestingly, while self-seeded ScQ15R reactions yield three discernible fibre types, wildtype reactions seeded by ScQ15R are skewed toward one peak, suggesting that the ScQ15R fibre types vary in their ability to incorporate wildtype protein (Fig. 4b). Thus both *in vitro* and *in vivo* templating of wildtype protein with the Sc mutants appears to result in propagating changes in the prion state.

The view that a single protein can misfold into multiple distinct self-propagating forms and that the range of preferred conformations is sensitive to primary structure and perhaps cellular environment[7,13] may explain several common features of prion transmission barriers and strains. For example, transmission barriers emerge rapidly compared to the loss of prion forming ability during evolution[7,8,10-12]. This could be explained by the fact that each conformation must be self-propagating and that mutations which completely prevent amyloid formation are likely to be rare compared to changes that disfavour only a subset of fibre types or prion strain

phenotypes[23,24] (Fig. 3a and 4a). In addition, it has been reported that crossing a transmission barrier can result in a change in the predominant prion strains. Because conformations vary in their specificity, the transmission barrier could act as a sieve, selectively amplifying infectious forms compatible with the recipient prion sequence[7,25,26]. More generally, the degeneracy in amyloid formation may provide a means of mitigating the toxic effects of protein misfolding: therapeutic strategies designed to promote formation of non-toxic conformations rather than preventing amyloid formation altogether may be more tractable, and evolutionary pressure may favour sequences that form less toxic conformations when they do misfold. Finally, the failure to create infectious forms of the mammalian prion protein (PrP) *in vitro* could be due to the preferential formation of non-infectious, self-propagating conformations outside the proper cellular context[27-29].

Methods

Plasmid construction and yeast strain construction

Inducer plasmids expressing Sc, Ca and Ch prion domains fused to GFP under the control of inducible *CUP1* promoters were described previously[8,21,22]. Mutant Ch inducers were generated via oligo-directed PCR mutagenesis. ScS17R also contained an R98H substitution and ScQ15R also contained R98H and N93D substitutions. Yeast strains expressing *SUP35* with the indicated prion domains were generated by replacing the wildtype chromosomal locus in the parental 74-D694 background as described previously[21].

In vivo analysis of prion strains and specificity

Efficiency of prion induction was determined by growing yeast strains harbouring appropriate inducer plasmids in synthetic media containing 5μM Cu_2SO_4 for 24 hours, plating on media lacking adenine, and counting colonies after four days of growth and normalized by plating an appropriate dilution of cells on synthetic complete media and counting after four days growth. Plates shown in Fig. 3d represent 8 days of growth illustrating that specificity of induction is not dependent on duration of growth. Phenotypic strength of induced prion strains were assayed by colony colour[21] on low adenine, non-selective YEPD media.

Amyloid formation assays

Prion domains C-terminally tagged with 7x (Sc) or 9x (all others) poly-histidine were produced as previously described[8]. Conversion to amyloid was monitored by a

continuous thioflavin-T based assay. Concentrated stocks of Sup35 variants stored in denaturant were diluted at least 50-fold into 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4, and 100 µl was added to 100 µl of 25 µM thioflavin-T (Sigma) in 50mM glycine, pH 8.0. Where indicated, reactions also contained 5% wt/wt of the appropriate sonicated amyloid seed. N.b., the efficiency of the seed is dependent on fibre fragmentation, which can vary from preparation to preparation. However, for a given seed preparation, initial rates of polymerization are linearly proportional to the amount of added seed (S.R.C, unpublished observations), and specificity, as determined by the relative rates of polymerization of two different monomers for a given seed, is therefore independent of the amount or degree of fragmentation of seed. Use of initial rates of polymerization also eliminates the effect of secondary nucleation events such as fibre breakage and *de novo* seed formation. Fluorescence was monitored in a 96-well fluorescence plate reader (Molecular Devices; 442nm excitation and 485nm emission). Thioflavin-T assays were carried out at 25 C and read automatically every minute with one second shaking between measurements. Single filament assays were performed as described[2] with the exception that here seeds produced by spontaneous polymerization were used directly, without passage through multiple rounds of polymerization and reseeding. Thermal stability of fibres (Fig. 1c) was determined by incubation of fibres at increasing temperatures (25 C to 95 C in ten degree intervals) for five minutes in 1.6 % SDS, followed by SDS-PAGE analysis.

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Figure 1.

Conformation of Ch fibres is sensitive to polymerization conditions. **a**, Amyloid polymerization curves monitored by thioflavin-T fluorescence. Ch fibres were used to seed Sc or Ca monomer as labeled. The Ch fibres were generated either by spontaneous polymerization at the indicated temperature or by templating with an Sc or Ca seed as indicated in brackets. **b**, Initial rates of polymerization from data in **a** (at least two independent measurements). **c**, Thermal stability of the indicated Ch fibre types as determined by SDS-PAGE (see Methods, Fig. S3). Total recoverable protein level was determined by boiling samples (final lane).

Figure 2.

Mutations create a transmission barrier *in vitro*. **a**, Schematic of model wherein mutations favour formation of Ch[Ca] state by specifically slowing the Ch[Sc] pathway. **b-d**, Specificity of seed as determined by initial rates of polymerization of the indicated monomer. In **b** and **c**, seeds were formed by spontaneous polymerization (at 25 C) of the indicated Sup35 prion domain. In **d**, seeds were produced by templating Ch with the fibres indicated in brackets. The difference in specificity between Ch (**c**) and Ch[Ch] (**d**) is most likely due to differential amplification of the mixed population of seeds generated during Ch polymerization.

Figure 3.

Mutations create a transmission barrier *in vivo* by shifting strain preference of Ch. **a**, Prion formation was induced in yeast expressing Ch Sup35 by transient overexpression of the indicated prion domain (top schematic). The relative fraction of 'strong' (gray) versus 'weak' (white) strains are shown below. **b-d**, Relative efficiency of prion induction following overexpression of the indicated prion domains in yeast expressing the indicated genomic Sup35 as assayed by growth on minus ADE media (see Methods). **e**, Initial rates of polymerization of indicated Sup35 prion domain with indicated seeds produced by either spontaneous polymerization of ChS17R or ChGA4 at 25 C (left) or by Ch fibres generated by templating with seeds indicated in brackets (right).

Figure 4.

Effects of mutations on the natural Sc Sup35 prion domain. **a**, Distributions of strain types (left) following induction of [*PSI*$^+$] in wildtype yeast (74-D764) by overexpression of Sc (black), ScQ15R (white), or ScS17R (gray) Sup35 prion domains. Examples of each strain category are shown at right. **b**, Distribution of polymerization rates of the indicated monomers onto individual preformed seeds (indicated in brackets) as determined by an AFM-based assay[2].

1. Dobson, C. M. The structural basis of protein folding and its links with human disease. *Philos Trans R Soc Lond B Biol Sci* **356**, 133-45. (2001).

2. DePace, A. H. & Weissman, J. S. Origins and kinetic consequences of diversity in Sup35 yeast prion fibers. *Nat Struct Biol* **9**, 389-96. (2002).

3. Baxa, U., Speransky, V., Steven, A. C. & Wickner, R. B. Mechanism of inactivation on prion conversion of the Saccharomyces cerevisiae Ure2 protein. *Proc Natl Acad Sci U S A* **99**, 5253-60 (2002).

4. Glover, J. R. et al. Self-seeded fibers formed by Sup35, the protein determinant of [PSI+], a heritable prion-like factor of S. cerevisiae. *Cell* **89**, 811-9 (1997).

5. Kad, N. M., Thomson, N. H., Smith, D. P., Smith, D. A. & Radford, S. E. Beta(2)-microglobulin and its deamidated variant, N17D form amyloid fibrils with a range of morphologies in vitro. *J Mol Biol* **313**, 559-71. (2001).

6. Lashuel, H. A., Hartley, D., Petre, B. M., Walz, T. & Lansbury, P. T., Jr. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature* **418**, 291 (2002).

7. Collinge, J. Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci* **24**, 519-50 (2001).

8. Santoso, A., Chien, P., Osherovich, L. Z. & Weissman, J. S. Molecular basis of a yeast prion species barrier. *Cell* **100**, 277-88 (2000).

9. Come, J. H., Fraser, P. E. & Lansbury, P. T., Jr. A kinetic model for amyloid formation in the prion diseases: importance of seeding. *Proc Natl Acad Sci U S A* **90**, 5959-63 (1993).

10. Chernoff, Y. O. et al. Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein. *Molecular Microbiology* **35**, 865-76 (2000).

11. Kushnirov, V. V., Kochneva-Pervukhova, N. V., Chechenova, M. B., Frolova, N. S. & Ter-Avanesyan, M. D. Prion properties of the Sup35 protein of yeast Pichia methanolica. *Embo Journal* **19**, 324-31 (2000).

12. Nakayashiki, T., Ebihara, K., Bannai, H. & Nakamura, Y. Yeast [PSI+] "prions" that are crosstransmissible and susceptible beyond a species barrier through a quasi-prion state. *Mol Cell* **7**, 1121-30. (2001).

13. Uptain, S. M. & Lindquist, S. Prions as protein-based genetic elements. *Annu Rev Microbiol* **56**, 703-41 (2002).

14. King, C. Y. et al. Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 6618-22 (1997).

15. Derkatch, I. L., Chernoff, Y. O., Kushnirov, V. V., Inge-Vechtomov, S. G. & Liebman, S. W. Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae. *Genetics* **144**, 1375-86 (1996).

16. Kushnirov, V. V., Kryndushkin, D. S., Boguta, M., Smirnov, V. N. & Ter-Avanesyan, M. D. Chaperones that cure yeast artificial [PSI+] and their prion-specific effects. *Curr Biol* **10**, 1443-6 (2000).

17. Zhou, P. et al. The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. *Embo Journal* **18**, 1182-91 (1999).

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

19. Uptain, S. M., Sawicki, G. J., Caughey, B. & Lindquist, S. Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion. *Embo J* **20**, 6236-45 (2001).

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

21. Chien, P. & Weissman, J. S. Conformational diversity in a yeast prion dictates its seeding specificity. *Nature* **410**, 223-7. (2001).

22. DePace, A. H., Santoso, A., Hillner, P. & Weissman, J. S. A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion. *Cell* **93**, 1241-52 (1998).

23. Barron, R. M. et al. Changing a single amino acid in the N-terminus of murine PrP alters TSE incubation time across three species barriers. *Embo J* **20**, 5070-8. (2001).

24. Mastrianni, J. A. et al. Inherited prion disease caused by the V210I mutation: transmission to transgenic mice. *Neurology* **57**, 2198-205 (2001).

25. Peretz, D. et al. A change in the conformation of prions accompanies the emergence of a new prion strain. *Neuron* **34**, 921-32. (2002).

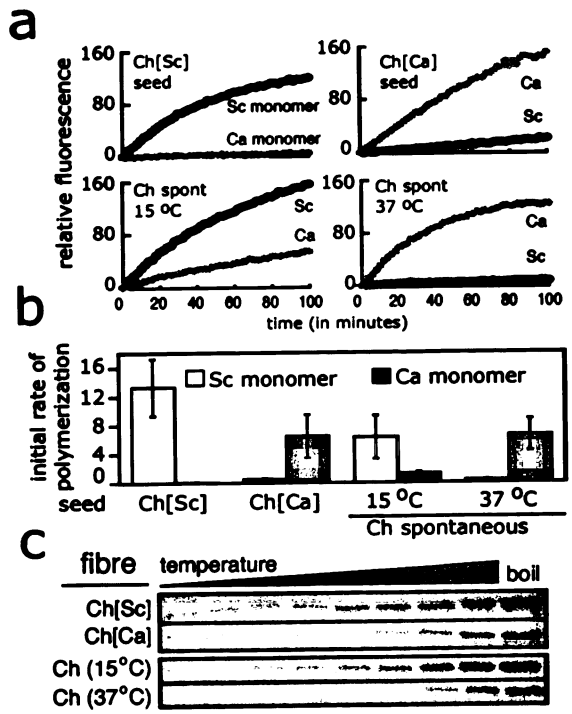
26. Bartz, J. C., Bessen, R. A., McKenzie, D., Marsh, R. F. & Aiken, J. M. Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. *Journal of Virology* **74**, 5542-7 (2000).

27. Hill, A. F., Antoniou, M. & Collinge, J. Protease-resistant prion protein produced in vitro lacks detectable infectivity. *J Gen Virol* **80**, 11-4. (1999).

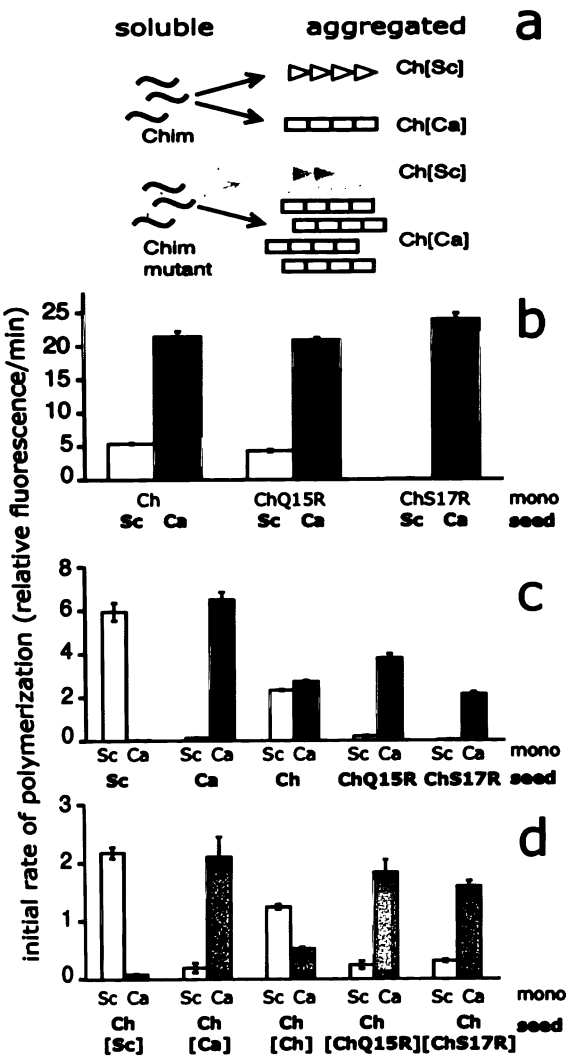
28. Baskakov, I. V., Legname, G., Baldwin, M. A., Prusiner, S. B. & Cohen, F. E. Pathway complexity of prion protein assembly into amyloid. *J Biol Chem* **277**, 21140-8 (2002).

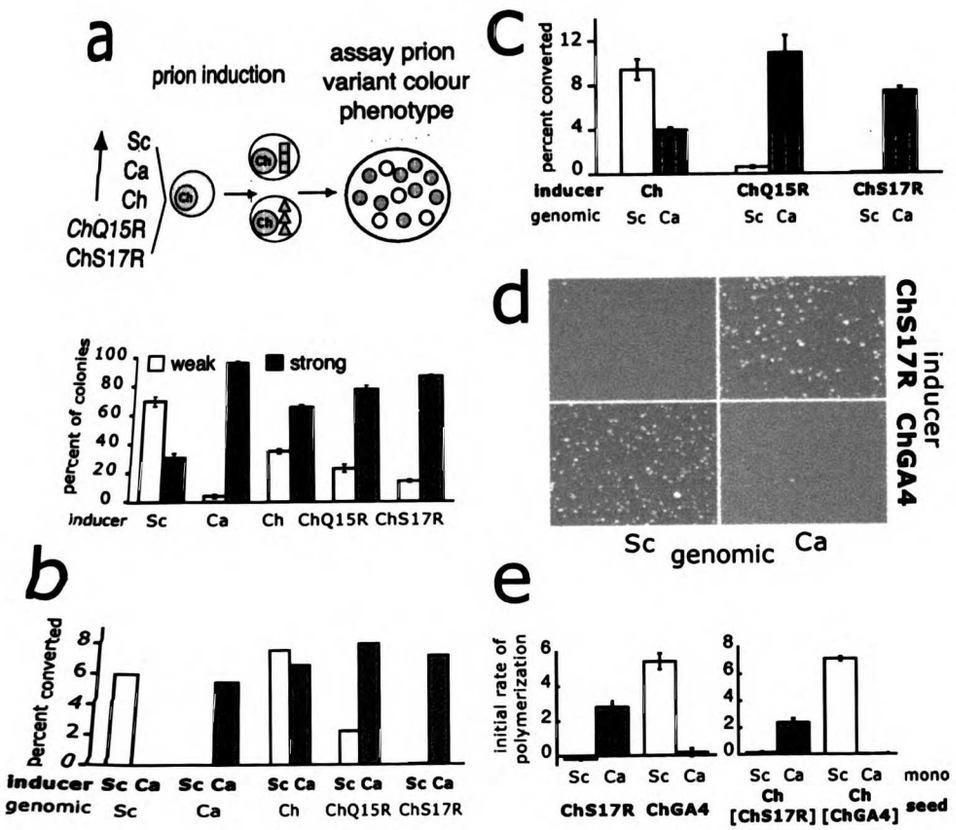
29. Post, K., Brown, D. R., Groschup, M., Kretzschmar, H. A. & Riesner, D. Neurotoxicity but not infectivity of prion proteins can be induced reversibly in vitro. *Arch Virol Suppl* **16**, 265-73 (2000).

Chapter 4
Figure 1



Chapter 4
Figure 2




a
prion induction
assay prion variant colour phenotype
Sc
Ca
Ch
ChQ15R
ChS17R
percent of colonies
weak
strong
inducer Sc Ca Ch ChQ15R ChS17R
b
Percent converted
inducer Sc Ca Sc Ca Sc Ca Sc Ca Sc Ca
genomic Sc Ca Ch ChQ15R ChS17R
c
percent converted
inducer Ch ChQ15R ChS17R
genomic Sc Ca Sc Ca Sc Ca
d
inducer ChS17R ChGA4
Sc genomic Ca
e
initial rate of polymerization
Sc Ca Sc Ca Sc Ca Sc Ca mono
ChS17R ChGA4 Ch [ChS17R] Ch [ChGA4] seed

Chapter4
Figure 4

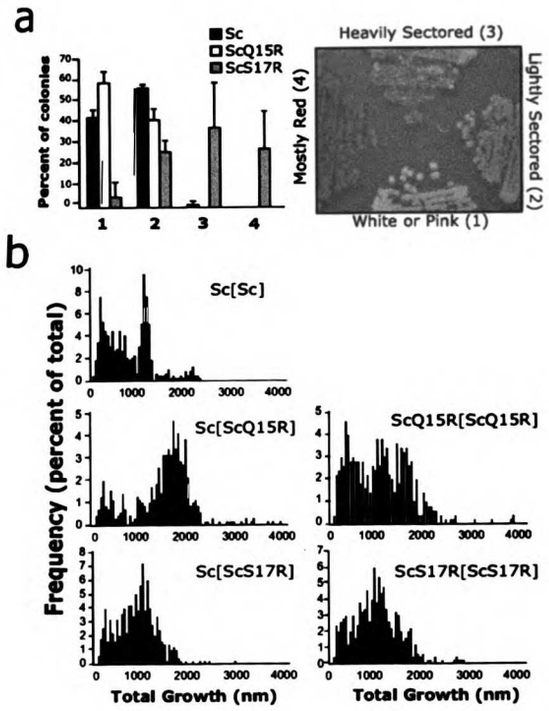
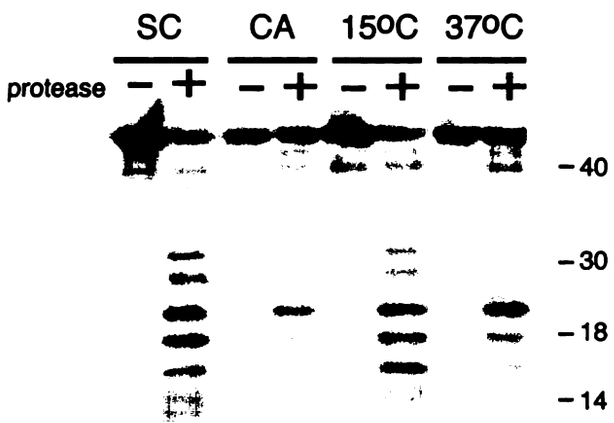


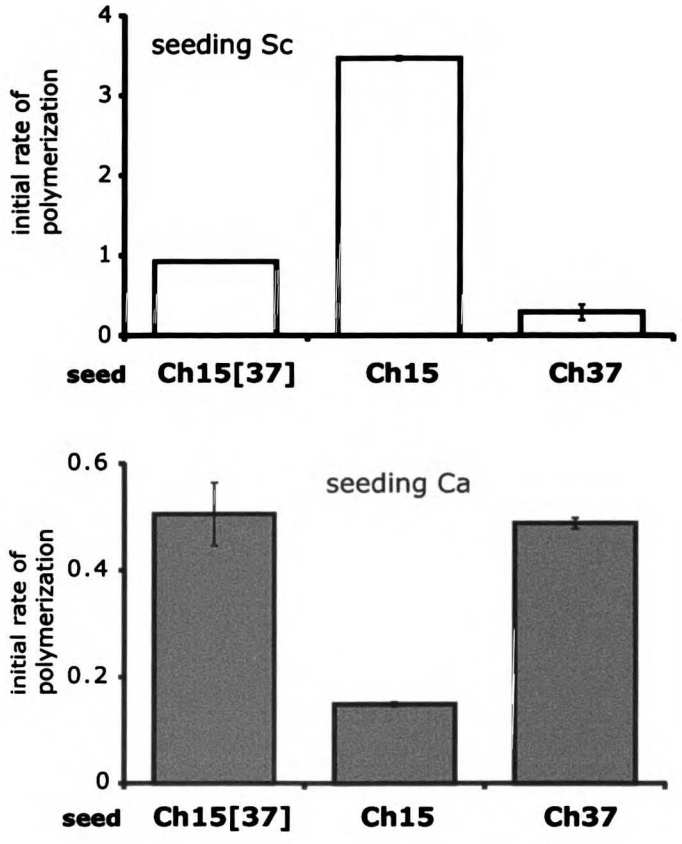
Table S1. Expansion and explanation of abbreviations

Abbreviation	Expansion	Explanation
	Prion domains	
Sc	*S. cerevisiae* SUP35 prion domain (including residues 1 - 253)	Prion domains are referred to by species and mutation where appropriate. (e.g., ScS17R is the SUP35 prion domain from *S. cerevisiae* with serine 17 replaced by arginine.)
Ca	*C. albicans* SUP35 prion domain (including residues 1 - 291)	
Ch	Chimeric prion domain (see supplemental data)	
	Fibre conformation	
Ch[X]	Ch fibres seeded by X	Fibre conformations are referred to by the fibre protein followed by its seed in brackets. (e.g, Ch[Sc] are Ch fibres initiated by preformed Sc seeds)
Sc[X]	Sc fibres seeded by X	
	Yeast strains	
	Yeast expressing full length SUP35 with:	Yeast strains are denoted by the species of prion domain replacing the native SUP35 prion domain. (e.g., SUP35-Ch yeast have Ch replacing the normal SUP35 prion domain, but leaving the rest of the gene intact.)
SUP35-Sc yeast	Sc prion domain	
SUP35-Ca yeast	Ca prion domain	
SUP35-Ch yeast	Ch prion domain	

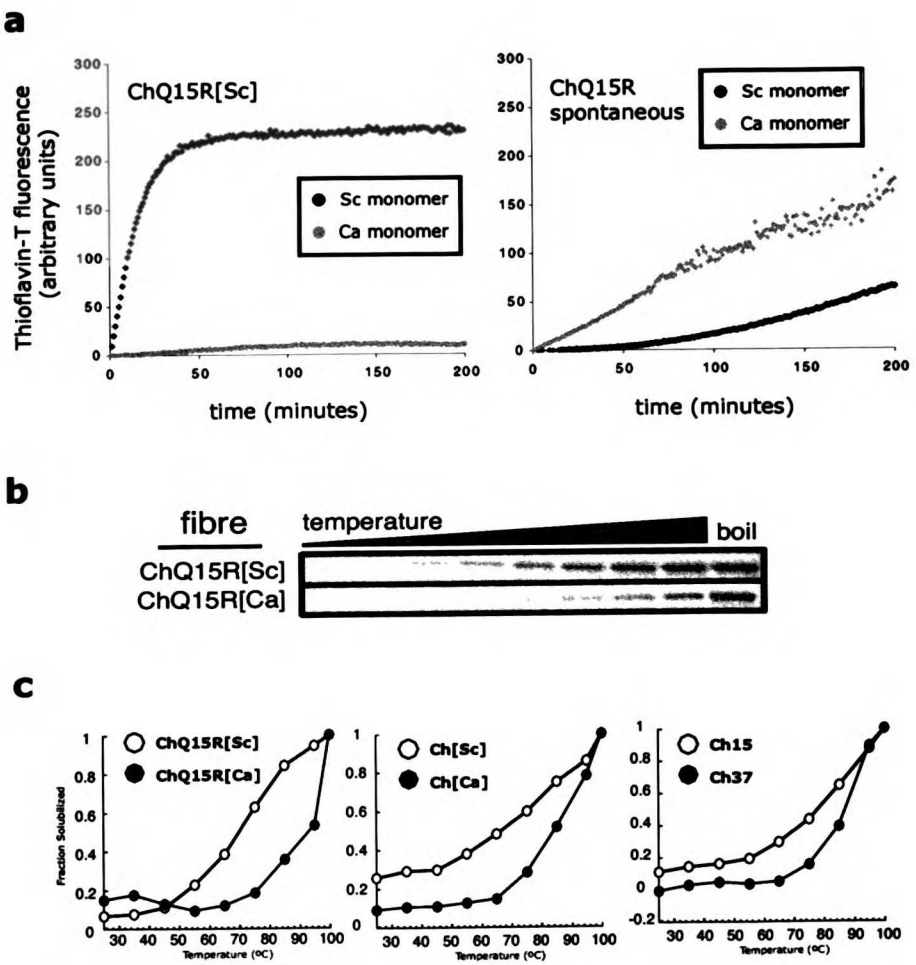
Supplemental Figure S1. Partial proteolysis of Ch fibres generated by seeding with indicated seed or by spontaneous polymerization at indicated temperature. Relative molecule weight (kDa) are indicated at right. In this experiment 3 micrograms of Ch fibres were incubated with or without chymotrypsin at 1:10 wt/wt for 5 minutes at room temperature. The reactions were quenched by addition of SDS loading buffer to obtain a final SDS concentration of 2% and boiled immediately for 10 minutes, then separated on a 4-12% Bis-Tris SDS-PAGE gel (Invitrogen). The gel was transferred to nitrocellulose, probed with anti-Sup35 antibodies21, imaged by chemiluminescence (SuperSignal West Pico substrate - Pierce), and documented with a FluorChem 8800 (Alpha Innotech).



Supplemental Figure S2. While Ch spontaneously polymerized at 15°C (Ch15) preferentially seeds Sc and Ch polymerized at 37°C (Ch37) favours Ca seeding, Ch polymerized at 15°C using Ch seeds polymerized at 37°C (Ch15[37]) retain seeding specificity of Ch37. Polymerizations were carried out as described in methods. Briefly, Ch fibres were rotated overnight at 37°C, sonicated the following morning and used to seed fibres at 15°C (5% wt/wt). In parallel, reactions without seed were rotated overnight at 37°C or 15°C. These resultant fibres were sonicated, and used as seeds in a thioflavin-T based assay to seed either Sc or Ca monomer as described.



Supplemental Figure S3. a. While ChQ15R spontaneously forms fibres which preferentially seed Ca, ChQ15R can be seeded by Sc to form ChQ15R[Sc], which specifically seeds Sc. **b.** Melting curve (see methods) of ChQ15R seeded by Sc or Ca. **c.** Quantitative densitometry (ImageQuant) of gels in **b** and in Figure 1C.



Supplemental Figure S4. Amino acid sequence of Ch prion domain. Residues mutated in ChQ15R (red), ChS17R (blue), and ChGA4 (green) are indicated. The Ca-derived region is boxed.

MSDSNQGNNQQNYQ**Q**Y**S**QNGNQQQGNNRYQGYQAYNAQAQ
SFVPQGGYQQFQQFQPQQQQQQY**GG**YNQYNQYQ**GG**YQQNYNNRG
GYQQGYNNRGGYQQNYNNRGGYQGYNQNQQYGGYQQYNSQPQQ
QQQQQSQGMSLNDFQKQQKQAAPKPKKTLKLVSSSGIKLANATKK
VGTKPAESDKKEEEKSAETKEPTKEPTKVEEPVKKEEKPVQTEEKTEE
KSELPKVEDLKISESTHNTNNANVTSADALIKEQEEEVDDEVVND

Chapter 5

A screen for suppressors of *C. albicans* SUP35 derived prions.

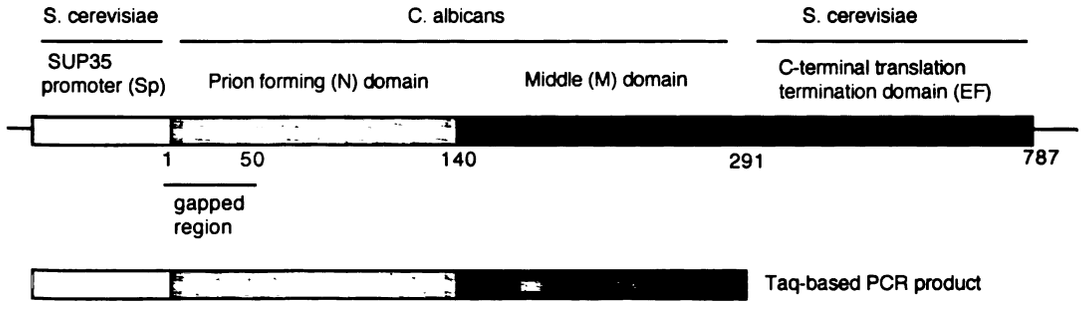
Chapter 5.

Mutational screen of *C. albicans* prion domain

The yeast prion [*PSI*$^+$] is a genetic element that is transmitted between cells through self-propagating aggregates of the translation termination factor Sup35p. This aggregation is mediated by an amino-terminal glutamine/asparagine-rich region that is necessary and sufficient for prion formation. A previous genetic screen in *Sacchromyces cerevisiae* (DePace and Santoso, et al 1998) has illustrated the critical nature of these polar residues in this region, demonstrating that even single point mutations can result in a debilitation of prion formation, alleviating the translation suppression phenotype (these mutations were denoted ASU - for anti-suppressed). Further work demonstrated that the SUP35 genes from other budding yeast could also form infectious propagating amyloid-based forms (Santoso, et al 2000). To assess whether similar critical regions were present in other species of Sup35p prion domains, we conducted a non-biased screen for mutations of the *Candida albicans* SUP35 prion domain that were deficient for interactions with wildtype *C. albicans* SUP35-based prion elements. We found that similar to the S. cerevisiae case single mutations could result in prion domains deficient in interacting with preexisting prion aggregates. Surprisingly, we repeatedly isolated mutations in a polyglutamine tract of a single residue truncation that showed a strong ASU phenotype. This is especially of interest as recent work (Resende, et al 2003) has shown that this polyglutamine region of C. albicans SUP35 is highly polymorphic between isolates, suggesting that prions from different isolates may not be able to robustly interact with each other.

The current mutational screen was performed by first amplifying the *C. albicans* prion domain with the S. cerevisiae SUP35 native promoter using Taq polymerase (which has an intrinsic error rate of about 10^{-3} basepairs). These PCR products were then co-transformed into yeast with a gapped version of 316SpCAEF plasmid (see Figure 1) that was missing the majority of the prion domain. After gap-repair by homologous recombination, these plasmids were selected for the ability of colonies to sector to red on low-adenine media. These positive clones were restruck onto both low-adenine selective media (without uracil) or rich media low in adenine to verify the colour phenotype. The plasmids were isolated using smash-and -grab followed by electroporation and retransformation to verify the phenotype.

Figure 1. Schematic of organization of 316SpCAEF with gapped region indicated.



The first class of mutations were similar to those found previously in the *S. cerevisiae* SUP35-based screen (Figure 2). Point mutations from polar residues to charged residues

were often seen, including those glutamine/asparagines to lysine/arginine mutations seen to be especially prevalent in the first screen. This suggests that the general correlation between asparagines and glutamine content, especially in the context of replacement by charged residues, is also seen in this species background. However, unlike the original screen, we did not uncover mutations mapping to a specific subregion of the prion domain. This data has two possible interpretations: One, that the prion forming capacity of the *C. albicans* prion domain is more generally distributed than that of the *S. cerevisiae*. Furthermore, as the especially critical far amino-terminal region of *S. cerevisiae* Sup35p was found to be a primary determinant of specificity (Osherovich, et. al. unpublished, DePace and Santoso, et al 1998, Chien and Weissman 2001, Crist, et al 2003) the lack of a parallel region in *C. albicans* Sup35p argues that this prion's specificity may not reside or be especially dependent on a single subregion epitope. The second interpretation is that the screen was incomplete and not performed to the point where specific regions become clear 'hotspots' for prion determination. However, if this is the case, it is surprising that we discovered multiple instances of mutations from independent PCR pools (especially those in the polyglutamine stretch).

One of the unique features of the *C. albicans* prion domain setting it apart from that of the S. cerevisiae Sup35 prion domain is that it contains a fairly long polyglutamine stretch (residues 24-34 in Figure 2. This stretch is highly polymorphic between isolates of C. albicans, as was shown in a recent report by the Tuite laboratory (Resende, et al 2003). In this study, the authors found that this stretch existed in all *C. albicans* SUP35 tested, but was either 7, 9, 11, or 13 glutamines in length. The codon usage is not

identical for the entire glutamine stretch, arguing that this tract has been retained even in the context of genetic drift. In our original wildtype *C. albicans* SUP35, there were 11 glutamines and in the course of our screen we consistently found ASU's that varied from the wildtype sequence only in this region, truncating the 11 glutamines to 10. This finding suggested that a single truncation of a residue in a larger glutamine tract could dramatically affect a prion protein's ability to be incorporated into the native aggregate and that aggregation of a pure polyglutamine stretch may have more structural components than previously thought.

Figure 2. Sequence of wildtype *C. albicans* Sup35p prion domain and ASU mutations found in the current screen.

C. albicans SUP35 prion domain (N)

msdqqntqdq lsgamanasl ngdqskqqqq qqqqqqqnyy npnaaqsfvp
qggyqqfqqf qpqqqqqqyg gynqynqyqg gyqqnynnrg gyqqgynnrg
gyqqnynnrg gyqgynqnqq yggyqqynsq pqqqqqqqsq g

ASU mutations

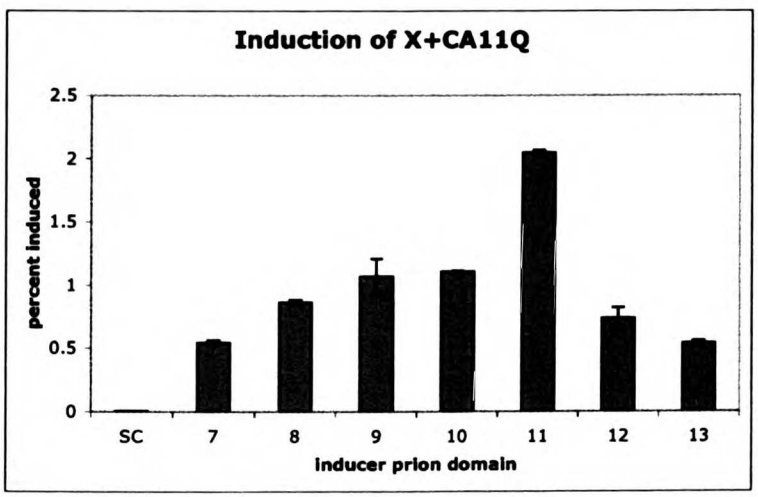
pool 3	*mutation*	*pool 4*	*mutation*	*pool 5*	*mutation*
3101	Q24P	**4101**	M1V	**5101**	deltaQ
3103	Q47R	**4102**	deltaQ	**5102**	Q46R
3104	Q27P	**4103**	deltaQ	**5103**	F57S
3106	M1V	**4104**	Q36R	**5106**	Q27R
3107	S47P	**4105**	Q29R	**5107**	D23G
3108	S47P	**4107**	deltaQ	**5108**	Q94L
3110	Q31R	**4108**	Q63R	**5110**	Q35R
3113	Q27R	**4111**	F57S	**5111**	F57S
3114	Q46R	**4112**	G22E	**5112**	Q27R
3115	Q77R				
3117	L20W				
3119	A14T;Q29P				
3120	Q30P				
3121	deltaQ				
3122	Q19R				

We sought to extend these findings by using a series of truncation and expansion mutants localized to this polyglutamine stretch. Through oligo-directed PCR mutagenesis we made mutants with 7, 8, 9, 10, 11, 12 and 13 glutamines in this stretch (to be known as CA7Q - CA13Q from this point on). These mutants were tested for their abilities to induce, maintain and cure prion states of the wildtype (CA11Q) SUP35 gene. We found that while all mutants could induce a prion state in CA11Q yeast upon transient overexpression, the CA11Q prion domain showed the most robust response (Figure 3). Furthermore, when put into 'maintainer' plasmids that fused these prion domains to the C-terminal translation termination functional region, all displayed variegated abilities to properly maintain the prion state (Figure 3). This suggested that although not generating the level of specificity seen with the species barrier limiting transmission between prion domains from different species, deletions or expansions of the polyglutamine tract in the

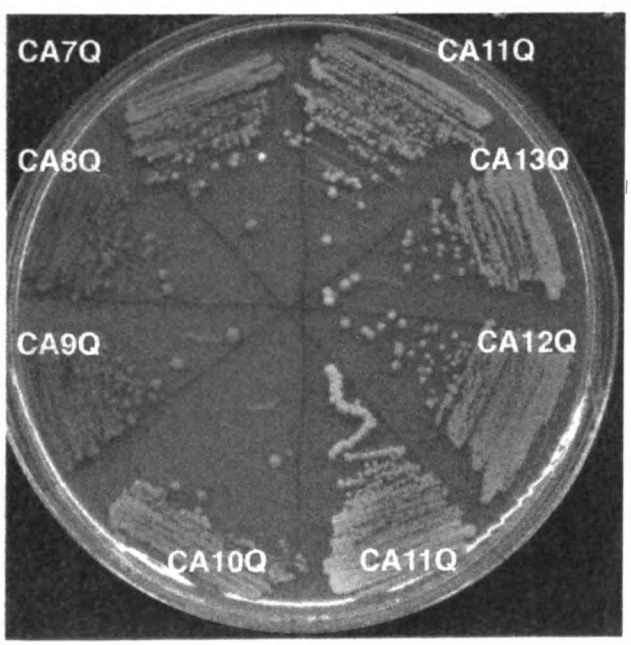
C. albicans prion domain can strongly modulate the ability to induce or maintain prion elements.

Figure 3. a) Induction of wildtype (CA11Q) transgenic yeast (see Chien and Weissman 2001) by transient overexpression of polyglutamine tract-mutant prion domains. b) Mutants (fused to C-terminal translation termination domain) show variegated abilities to be recruited into preexisting CA11Q prion aggregates.

a



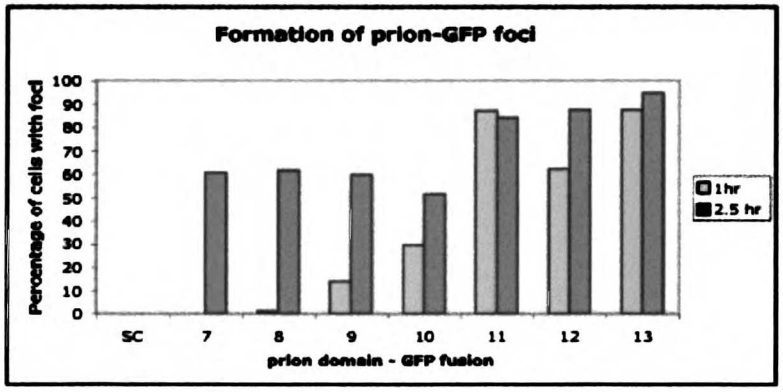
b



The variegated ability to maintain the CA11Q prion state was surprising, especially when considering that these mutants have both an expanded, i.e., CA12Q or CA13Q, and truncated polyglutamine tract. We tested the aggregation of these mutant prion domains when fused to GFP as a reporter in the context of the CA11Q prion background. The truncated prion domains consistently formed aggregates at a slower rate when compared to the 'native' (CA11Q) or polyglutamine expanded mutants (Figure 4). Interestingly, after plating these yeast onto non-selective low adenine media, we found a correlation with this aggregation profile and curing of the preexisting CA11Q prion aggregate phenotype. Precedence for this behaviour has been established as previous work had shown that [*PSI*$^+$] can be cured by overexpression of the Sup35p prion domain (King 2001) and to the extreme, massive overexpression of the prion domain in [*PSI*$^+$] yeast has a somewhat lethal effect. However, the degree and timescale of this curing has not been well-examined, therefore we examined in detail the number of cured colonies as a function of time post-induction. These mutant prion-GFP fusions are driven by the Gal promoter, which allows us to rapidly turn on and turn off expression by inducing with galactose and switching to glucose for each timepoint upon plating. Remarkably, curing of X+CA11Q yeast was seen after just one hour of induction. This suggests that *i*) minute amounts of mutant prion domains are sufficient to initiate permanent curing and *ii*) this initiation is not strictly dependent on cell-division since after an hour in this media, no significant cell-division has occurred.

Figure 4. a) Aggregation of mutant prion domains fused to GFP in a X+CA11Q background. b) Timecourse of curing of X+CA11Q yeast by mutant prion - GFP fusions (SC represents *S. cerevisiae* SUP35 prion domain control). Expression of GFP fusions was initiated with galactose, the timescale reflects number of hours of induction before plating on dextrose media.

a



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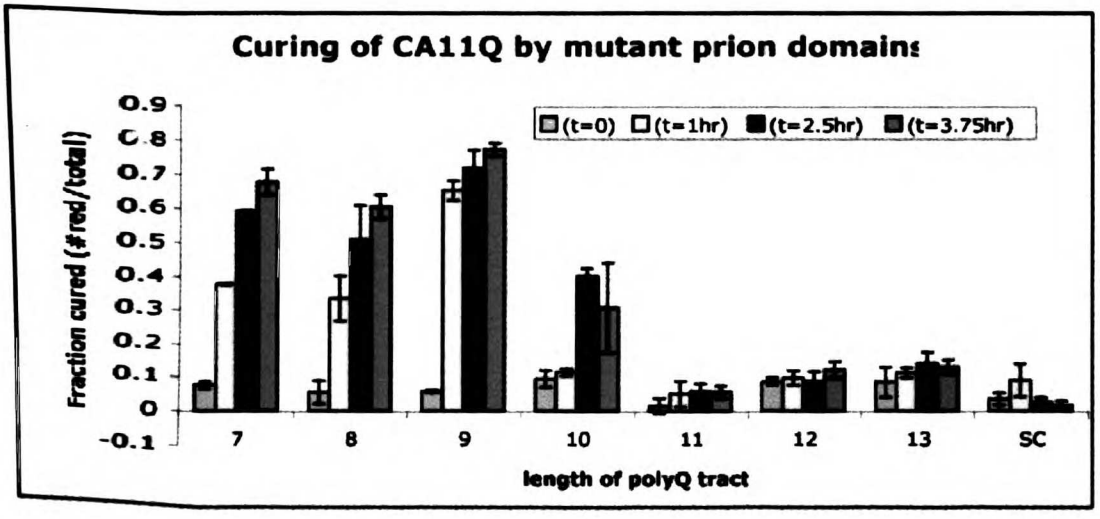


References

Chien P, Weissman JS. 2001. *Nature* 410: 223-7

Chien P, DePace AH, Collins SR, Weissman JS. 2003. *Nature* 424: 948-51

Crist, C.G., Nakayashiki T., Kurahashi, H., Nakamura, Y. 2003. *Genes Cells.* 8: 603-618

DePace AH, Santoso A, Hillner P, Weissman JS. 1998. *Cell* 93: 1241-52

Resende C, Parham SN, Tinsley C, Ferreira P, Duarte JA, Tuite MF. 2002. *Microbiology* 148: 1049-60

Santoso A, Chien P, Osherovich LZ, Weissman JS. 2000. *Cell* 100: 277-88

Chapter 6

Protocols and Unpublished Experimental Observations

UNPUBLISHED OBSERVATIONS AND PROTOCOLS

Census of glutamine/asparagines content of ORFs from *S. cerevisiae, C. albicans*, and *S. pombe*.

These observations began with the generation of a computer program written in PERL called DIANA, originally conceived by Jonathan Weissman and Melissa Michelitsch and published in Proceedings of the National Academy of Sciences in 2000. This program was to sift through published open reading frames of sequenced genomes and determine content of glutamine and asparagines residues by moving an 80-mer window through the sequence and determining glutamine/asparagines (Q/N) content. An ORF containing an 80-mer window with greater than either 30 or 35 Q/N's was then considered a potential prion domain-containing candidate.

I rewrote the program in 2002 in order to make it a bit more user-friendly and commented. The program is written in PERL and follows this paragraph. I used this program to look through published sequences for C. albicans, S. cerevisiae and S. pombe (a fission yeast). The results were quite interesting as C. albicans and S. cerevisiae containing a similar number of ORFs with relatively (30 or more) high Q/N content within a given 80-mer window. C. albicans had 195 out of about 8000 ORFs, while S. cerevisiae had 98 out of about 6000 ORFs. The surprise came when I looked at S. pombe, which had 5 out of about 4500 ORFs. Every yeast prion protein that was characterized in S. cerevisiae (such as SUP35, NEW1, RNQ1, or URE2) had a homologue in S. pombe, but was missing a 'canonical' Q/N rich prion domain. These data and the original genome files are copied onto a CD that should be located with my

files – labeled 'q_n_genomes'. Below is the example for SUP35 with the highly conserved C-terminal functional end of SUP35 emboldened:

S.pombe SUP35 (SPCC584.04)

MASNQPNNGEQDEQLAKQTSKLSMSAKAPTFTPKAAPFIPSFQRPGFVPVNNIAGGYPY
AQYTGQGQNSNSPHPTKSYQQYYQKPTGNTVDEDKSRVPDFSKKKSFVPPKPAIPKGKV
LSLGGNTSAPKSTKPISISLGGTKAPTTTKPAAPAAQSKTETPAPKVTSESTKKETAAP
PPQETPTKSADAELAKTPSAPAAALKKAAEAAEPATVTEDATDLQNEVDQELLKD**MYGK**
EHVNIVFIGHVDAGKSTLGGNILFLTGMVDKRTMEKIEREAKEAGKESWYLSWALDSTS
EEREKGKTVEVGRAYFETEHRRFSLLDAPGHKGYVTNMINGASQADIGVLVISARRGEF
EAGFERGGQTREHAVLARTQGINHLVVVINKMDEPSVQWSEERYKECVDKLSMFLRRVA
GYNSKTDVKYMPVSAYTGQNVKDRVDSSVCPWYQGPSLLEYLDSMTHLERKVNAPFIMP
IASKYKDLGTILEGKIEAGSIKKNSNVLVMPINQTLEVTAIYDEADEEISSSICGDQVR
LRVRGDDSDVQTGYVLTSTKNPVHATTRFIAQIAILELPSILTTGYSCVMHIHTAVEEV
SFAKLLHKLDKTNRKSKKPPMFATKGMKIIAELETQTPVCMERFEDYQYMGRFTLRDQG
TTVAVGKVVKILD

S. cerevisiae SUP35

MSDSNQGNNQQNYQQYSQNGNQQQGNNRYQGYQAYNAQAQPAGGYYQNYQGYSGYQQGG
YQQYNPDAGYQQQYNPQGGYQQYNPQGGYQQQFNPQGGRGNYKNFNYNNNLQGYQAGFQ
PQSQGMSLNDFQKQQKQAAPKPKKTLKLVSSSGIKLANATKKVGTKPAESDKKEEEKSA
ETKEPTKEPTKVEEPVKKEEKPVQTEEKTEEKSELPKVEDLKISESTHNTNNANVTSAD
ALIKEQEEEVDDEVVND**MFGGKDHVSLIFMGHVDAGKSTMGGNLLYLTGSVDKRTIEKY**
EREAKDAGRQGWYLSWVMDTNKEERNDGKTIEVGKAYFETEKRRYTILDAPGHKMYVSE
MIGGASQADVGVLVISARKGEYETGFERGGQTREHALLAKTQGVNKMVVVVNKMDDPTV
NWSKERYDQCVSNVSNFLRAIGYNIKTDVVFMPVSGYSGANLKDHVDPKECPWYTGPTL
LEYLDTMNHVDRHINAPFMLPIAAKMKDLGTIVEGKIESGHIKKGQSTLLMPNKTAVEI
QNIYNETENEVDMAMCGEQVKLRIKGVEEEDISPGFVLTSPKNPIKSVTKFVAQIAIVE
LKSIIAAGFSCVMHVHTAIEEVHIVKLLHKLEKGTNRKSKKPPAFAKKGMKVIAVLETE
APVCVETYQDYPQLGRFTLRDQGTTIAIGKIVKIAE*

As can be seen, while S.cerevisiae SUP35 has a Q/N rich N-terminal domain, the corresponding region from S. pombe SUP35 is slightly shorter and is not enriched in Q/N residues. This is similar to all examined known yeast prions.

I interpreted this lack of Q/N-rich regions in S. pombe as an indication that perhaps its chaperone machinery would be particularly intolerant of Q/N-based aggregation. In the same vein, the chaperone necessary for prion propagation in S. cerevisiae (Hsp104) may not have preserved this function when looking at the S. pombe homolog. I cloned the S. pombe HSP104 and found that while it could cover the essential thermotolerance function of the chaperone and could support propagation of [PSI+], this homolog could not cure [PSI+] when overexpressed. This work was and is being continued by Kimberly Tipton.

In addition I attempted to overexpress glutamine/asparagines rich regions in S. pombe to possibly detect a toxic effect. Overexpression of the C. albicans Sup35p prion domain fused to a C-terminal GFP showed no effect on cell growth and did not form foci in S. pombe. These vectors and methods are described in the "S. pombe notebook" in my records.

THE 'DIANA' program:

```
#DIANA program for Q/N content within 80mer window in ORF sequence
#this opens a fasta format file with orf sequences
#you would put in the path to your filename in the quotations
#for instance:  "\home\fastafiles\myfile.txt"
#make sure not to delete the semicolon

$fastafile = "YOUR-PATH-GOES-HERE";

open FILEH, $fastafile or die "no open \n";

while(!(eof FILEH)){

#this section reads in a file line by line to look
#at individual orfs

while (<FILEH>) {
      if (/^>/) {
$wttnext = $_;
last;}
```

```
      $wtt = $wtt.$_;
}
$wtt1 = $wtt;

#this command splits the > header from the
#sequence of the orf itself

($header,$seq) = split(/\n/, $wtt1,2);

$seq =~ s/\n//g;

#this throws away any orf under 80 amino acids long

$orfsize = length $seq;
if ($orfsize < 80) {next;}

$i = 0;
$ctold = 0;


#this moves a window of 80-mer along the entire polypeptide
#and counts number of glutamines and asparagines
#and gets the max number of gln/asn for any given 80-mer

while ($i < ($orfsize)) {
$a = substr $seq, $i, 80;

$qct = $a =~ s/Q/q/g;
$nct = $a =~ s/N/n/g;

$ct = $qct+$nct;

if ($ct > $ctold) {$ctold = $ct; $tempbuff = $a};

$i++;
}


#this sets the cutoff - it will output all orf headings with gln/asn
#greater than 30 in ANY 80-mer in that polypeptide
#it prints an index number (starting with zero or blank)
#then the max value of gln/asn (out of 80-mer window) for that orf
#then the header of the fasta entry

if ($ctold > 30) {

print $j."-".$ctold."-".$header."\n";

$j++;}

$wtt = $wttnext;
$k++;
}
```

Protocol for spindown assay

```
SPIN DOWN PROTOCOL (Alex's)

Culture
Grow 50 ml culture to OD600  0.5 - 1
Spin down and wash with 25 ml water
Resuspend in 1 ml Buffer A and Protease Inhibitors (cold)
<\optional>
Add 4 ul 0.5 M betaME / 50 ul lyticase
Incubate at room temp w/ gentle rotation 30 min
</optional>
Add 250 ul beads
Turbomix 5-10 min
Spin 8000 rpm @ 4 degC 3 min
Save SUP

100,000 Spin
Load 100 ul onto ultracentrifuge tube
30 min 100K 4 deg C
Save SUP and PELLET (take 50 ul off top to save, dump rest
to prevent contamination of fractions)
Resuspend pellet in 100 ul Buffer A
*in theory, can add SDS running buffer here, and store
overnight
have had mixed luck personally*

Run Gel
Use Biorad to check [protein]
load 20 - 40 ug, load same volume for pellet
Do western


Buffer A
25mM TrisCl pH 7.5
50 mM KCl
10 mM MgCl2
1mM EDTA
5% glycerol
must add: 1mM PMSF,2ug/ml pepstatin and leupeptin, 100
ug/ml RNAseA
```

Protocol for denaturing purification of prion domains

Sup35NM(his-tag) purification:
Peter Chien's protocol - <pchien@itsa.ucsf.edu>

Growing up cells:

Day One:
1. Transform plasmid into BL21 cells (you don't need to worry about pLysS)
and plate onto LB Carb or Amp plates - this should be done in early
afternoon, around 3-5 pm so as to give cells plenty of time to grow.
You want to transform and plate enough cells to get 100 - 1000 colonies
coming up the next day. I use one plate for every Liter of cells I want to grow.

Day Two:
2. Next day (early in morning) scrape plates. This can be done either by
physically scraping the top of the plates with a metal spatula - must be done
carefully!!! -or- add 250 - 500 microliters of water to the plate and either use
glass beads or a spreader to suspend colonies. Either way, inoculate 50 ml of
LB/Carb in a 250 ml flask with all the cells. (for instance if I have six plates - planning on growing six liters - I still inoculate only 50 ml with all six plates worth of cells). Let these shake at 37 degrees C for about an hour.

3. Innoculate 1 L LB/Carb with appropriate amount of culture - if you want to
grow 6 liters, and have scraped 6 plates, then you should add 8.3 milliliters of
the small culture to each liter. Shake at 37 degrees C.

4. When the cells get to OD600 of 0.3 - 0.6 , take a 200 microliter aliquot and
add 0.4 mM IPTG into the cells (you can get away with 0.2 mM, but I generally
add more than needed). It's pretty important to keep the cell density at this
somewhat lower than normal level for induction. (Sup35NM is somewhat toxic

to bacteria and when overexpressed tends to kill cells. This is why an overnight
culture can't be used reliably as a starter for larger cultures, the cells that do
come up have learned to downregulate Sup35NM.)

5. Let induce for anywhere between 2 hrs and 4 hrs. Harvest the cells in 1 liter
flasks, wash once with water, and freeze at -80 degrees C.

Ni-NTA purification

Buffer A: 8 M Urea, 25 mM Tris, 300 mM NaCl, pH 7.8

Buffer B: same as A, but 0 mM NaCl

Buffer C: same as B, but pH 4.5-5

*to be honest, Tris doesn't buffer @ 4.5, so really, buffer C is not
strictly buffered, doesn't really matter though - can use NaPO4 buffer
as well instead of Tris, but then you have more salt .... up to you

1. Pour Ni-NTA gravity column; I use about 5 ml bed volume per L of cells; (each ml of resin should bind 5 mgs or so of protein), I routinely use a 20 ml (bed volume) column for all my purifications, seems to be more than enough resin.

2. Equil column w/ approx 5x column volume buffer A.

3. Add Buffer A to cell pellet - I use 25 ml / pellet (assuming 1 pellet = 1L culture)

4. Vortex to loosen and thaw pellet. Sonicate w/ microtip for approx 1 min to really break up pellet and hopefully get more of the inclusion bodies out.

5. Rotate/nutate/orbital shake for 30 min - 2 hrs @ room temp.

6. Transfer to 30 ml centrifuge tubes. Spin @ 30,000 x g for 20 minutes and save supernatent. (might need to spin longer depending on pellet)

7. This is the "fun" part. You have to preclear the lysate. This sucks. There are several methods that people have tried. The most arduous, but most reliable is to get a box of 0.45 um syringe filters and a 50 ml syringe. Fill the 50 ml syringe w/ lysate, screw on a 0.45 um filter and push. You'll get about 3-5ml of clear lysate before the damn thing clogs. Remove filter (watch out ! - there's pressure so might get lysate spewing!), replace with fresh one, do again. When AHD or PC do this, there is much cursing and talk of "we should really find a better way to do this" yelling. Eventually (approx 10 - 20 filters later, depending) the lysate is precleared.

8. Load precleared lysate to equilibriated column. Collect Flow through. Add 4x column volume of Buffer A to wash. Collect washes to check for protein. (see 11)

9. Wash with 4 x column volumes of Buffer B to get out salt.

10. Elute with several (3 - 5) 1 x column volumes of Buffer C to elute. Sup35NM usually elutes in first, second and third elutions, but sometimes weird things happen.

11. Run fractions (approx 5 ul each) on 10 % SDS-PAGE to assess purity. Pool and save fractions in liq. N2 , store @ -80. Remember that Sup35NMhis will run at higher than you would expect from the sequence - it's supposed to be 29 kDa, but runs at around 45 kDa for us alot of times.

n.b on step 7: I've (PC) used a coffee filter sometimes to pre-preclear the lysate. Sometimes makes it a bit easier to syringe filter later. Be sure to use NON-BLEACHED (i.e, brown) coffee filters, cause bleach is bad for protein too.

**alternative to preclearing lysate - use the following instead of steps 7 and 8:

7a. Instead of preclearing the lysate, use a batch method to do the initial purification. Take 20 ml of Ni-NTA resin (from the bottle, will be in 50% EtOH). Spin in 50 ml centrifuge tube for 30 seconds at 500 x g. Wash the resin this way (remove supernatant, resuspend resin, spin again) at least two or three times with water to make sure that the EtOH has been removed. Now, wash with Buffer A two or

three times in same fashion to equilibriate the resin in Buffer A.

8a. Add lysate to the equilibriated resin, making sure to resuspend the resin. Rock or rotate this mixture at room temperature for 30 minutes - 1 hr. Pour the mix into a empty column and collect the flow through. Add 4x column volume of Buffer A to wash. Collect washes to check for protein. (see 11)

**these alternative steps are faster, but might make it harder to reuse the resin (because a lot of cell debris that was in the lysate might clog the resin and make it bad to use for future purposes)

SourceS or ResourceS:
Sup35NMhis after the initial Ni-NTA column is usually pretty clean, but to get it really clean we run the protein over a sourceS or ResourceS column (any cation exchange column should work,but you might have to change the gradient conditions.) This purification is all done with an FPLC at room temperature.

Buffer A.
8 M Urea
50 mM MES
pH 6.0

Buffer B.
8 M Urea
50 mM MES
1 M NaCl
pH 6.0

all flow rates are kept at 1 ml/min

1. Equilibriate column in Buffer A.

2. Load the protein-rich elutions from the Ni-NTA column. We routinely load up to 30 ml of protein onto a 6 ml ResourceS column, but this should be checked according to your system.

3. Wash with Buffer A alone for 2 - 3 column volumes.

4. Use a 0-20% B gradient over 90 minutes for the elution. Sup35NMhis should elute around 10 % or so (around 40 minutes for our setup). Keep an eye on the A280 and A229 to see when the protein starts eluting. Collect 1 ml fractions. The elution profile will be quite a sharp increase at the beginning a bit of a tail. The really pure Sup35NMhis will be in the first park of the elution peak.

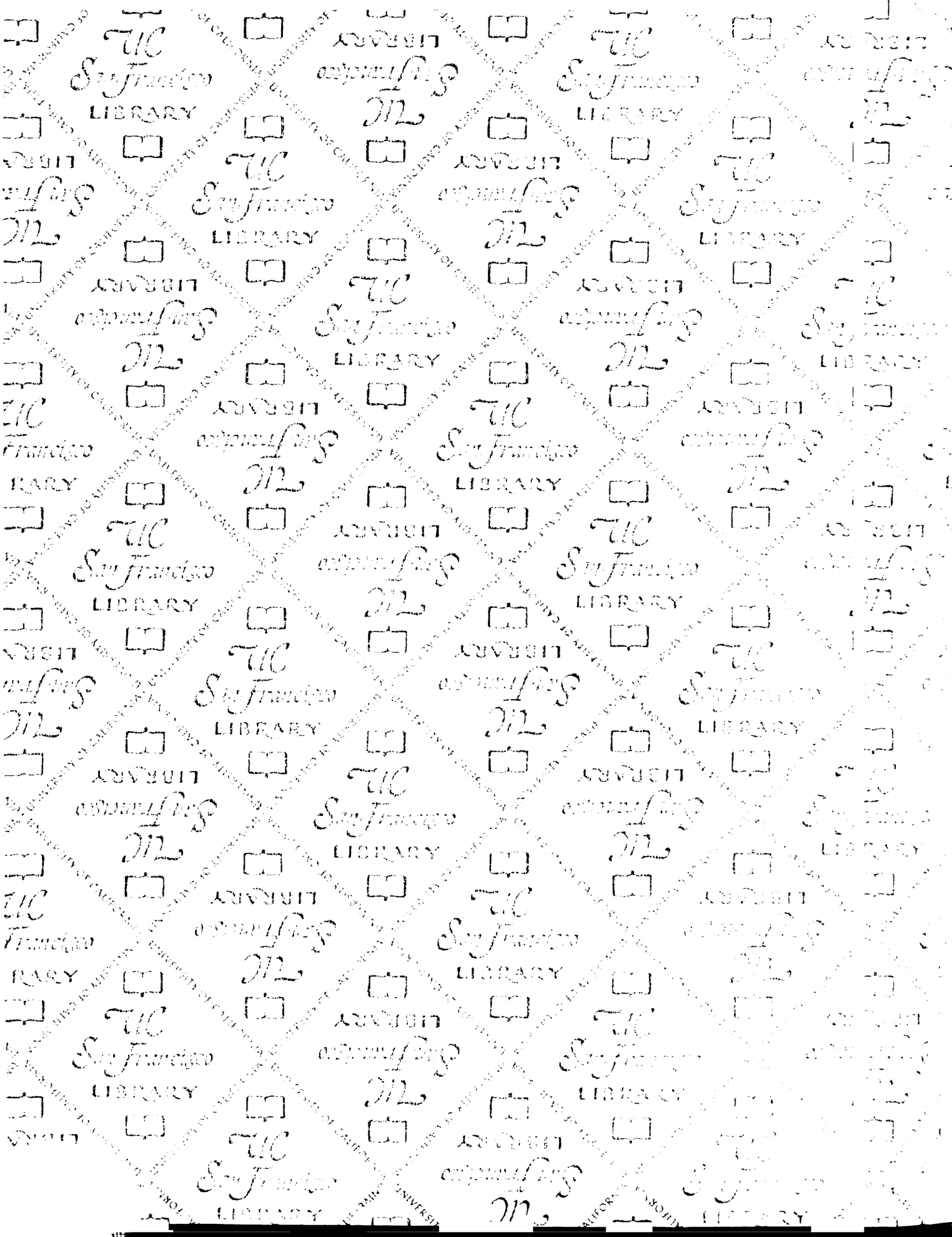
5. Run a 10 % SDS-PAGE gel on the fractions. You should see very pure protein in the first fractions, then more and more degradation products on following fractions. Pool the really pure fractions (we keep fractions that are >90% pure).

Concentrating the protein:
For most purposes we concentrate the protein so that we can dilute it to micromolar concentrations and keep denaturant concentration to a minimum. Typically this means concentrating the protein to about 500 - 900 micromolar (uM). We use 10 kDa spin concentrators (from Vivascience or from Millipore) to concentrate the protein to about 1 mM or so - you can check protein concentration by absorbance at 275 nm, assuming an extinction coefficient of 27,000 /mol/cm. After concentrating the protein you must filter it through a 100 kDa filter to remove any aggregates that may have arisen due to concentrating. We tend to lose quite a bit of material because of this (up to 50 %) but it's worth it to get pure MONOMERIC protein.

I find that if you add 6M Guanidine hydrochloride to the concentrated material and let it incubate at room temperature for an hour or so, you can recover more material from the 100 kDa filtration step. My method is to add enough 6M Gdn to the concentrated material to keep the Gdn concentration above 4 M, then let incubate for an hour at room temperature, then concentrate the material again with 10 kDa filters, then filter material through a 100 kDa filter.

I hope that these instructions are useful and will aid in purifying Sup35NMhis.

Remember that it is an aggregating protein so keeping it constantly in denaturant will improve yield. I find that GdnHCl is a much better denaturant for Sup35Nmhis unfortunately you can't use it all the time because of the ion exchange column and also because you can't run SDS-PAGE gels on Gdn containing samples. Good luck in purifying protein!!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