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Unraveling Infectious Structures, Strain Variants and Species Barriers for the Yeast Prion [*PSI*⁺]

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Abstract

Prions are proteins that can access multiple conformations, at least one of which is β -sheet rich, infectious and self-perpetuating in nature. These infectious proteins display several remarkable biological activities, including the ability to form multiple infectious prion conformations (also known as strains or variants) that encode unique biological phenotypes, and the ability to establish and overcome prion species (transmission) barriers. In this review, we highlight recent studies of the yeast prion [*PSI*⁺], using various biochemical and structural methods, that have begun to illuminate the molecular mechanisms by which self-perpetuating prions encipher these biological activities. We also discuss several aspects of prion conformational change and structure that remain either unknown or controversial, and propose approaches to accelerate the understanding of these enigmatic, infectious conformers.

Introduction

The “misfolding” and assembly of proteins into β -sheet rich, amyloid fibers is important in both disease¹ and normal biological function.^{2,3} Although many proteins form amyloid fibers *in vitro*, understanding the biological relevance and consequences of this process *in vivo* is difficult. Prions are one class of naturally occurring, amyloid-forming proteins that have received much attention.³⁻¹⁰ The first prion protein, PrP, was identified in mammals as an infectious agent responsible for several related neurodegenerative diseases, known collectively as the spongiform encephalopathies.^{8,10} How a protein could be infectious was a complete mystery until it was discovered that the protein in question was a normal constituent of the brain that simply changed its conformation from an α -helical to a β -sheet form to become infectious.⁸⁻¹⁰ Once this conformation appears in the brain – due to contamination by infectious material, spontaneous conversion or mutation-induced misfolding – it is self-templating, converting more and more PrP to the infectious form and wrecking havoc in the brain as it does so.⁸⁻¹⁰ Even so, it took many years for the “protein-only” mechanism of prion transmission to be accepted.

The discovery of a similar process operating in yeast cells, where it could be investigated more readily due to the ease of genetic manipulation, was an important factor in winning this battle.¹¹⁻¹³ The prions of yeast and other fungi consist of completely different proteins whose sequences are unrelated to their mammalian counterparts.^{3,4,6,11} Moreover, fungal prions are generally not deleterious and can even be beneficial.³⁻⁷ They serve as heritable elements, producing stable new phenotypes due to a profound change in protein conformation that is self-templating and transmissible from mother to daughter cells.^{3,4,6,11} Indeed, the recent proposal of a prion-like mechanism for the perpetuation of synapses and neuronal memories,¹⁴ as well as a host of new prions with diverse functions in yeast,^{15,16} indicates that prions will prove vitally important in many organisms.

An important similarity between mammalian and yeast prions is that they form not just one prion conformation, but a collection of structurally related yet distinct conformations, known as prion strains.¹⁷⁻

²³ For example, mice infected with prions from diverse animal origins manifested different patterns of

disease and these could be stably passed from mouse to mouse.²⁴⁻²⁸ Although a seemingly obvious explanation was distinct viral strains, an explanation independent of nucleic acid emerged as evidence mounted that these different diseases traced to different (yet related) self-templating folds of the same protein, PrP.²⁴⁻²⁸ Similarly, for yeast prions, unique protein folds produce a suite of distinct (yet related) prion phenotypes.¹⁷⁻¹⁹

Another critically important feature shared by mammalian and fungal prions is the species barrier.^{9,24,25,29-38} The aforementioned prion strains display extremely low prion infectivity when introduced into mice, yet once these mice succumbed to disease, mouse-to-mouse transmission was extremely efficient. Yeast prions also exhibit strong species barriers that can be crossed, but with difficulty.^{28-32,34,35,39-41} Remarkably, for both mammals and yeast, prion strains and species barriers are interrelated.^{4,8,9,24,26,27,29,37,40}

To decipher the complexities of these problems *in vivo*, it is necessary to analyze the biochemical properties of these proteins. Unfortunately, forming highly infectious mammalian prion conformers *in vitro* from recombinant protein has been difficult (for recent progress, see refs^{42,43}). In contrast, *bona fide* highly infectious fungal prion conformers can be readily formed *in vitro*,^{18,19,44-46} allowing a more thorough characterization of their assembly process and amyloid structure, which will be reviewed here.

Known and Potential Fungal Prions

The most well studied fungal prion proteins are Sup35, Ure2, Rnq1 and HET-s.³⁻⁷ Sup35 is a protein involved in recognition of stop codons during protein synthesis (Figure 1). Conversion of Sup35 from its soluble non-prion state, [*psi*⁻], to its aggregated prion state, [*PSI*⁺], causes reduced termination activity.^{12,47,48} This results in increased read-through of stop codons and reveals complex phenotypes that, in some cases, are beneficial.⁴⁹⁻⁵¹ Ure2 is an inhibitor of Gln3, a transcription factor that represses genes involved in metabolizing poor nitrogen sources when better ones are present.^{3-7,52} When Ure2 switches from its soluble non-protein state, [*ure-o*], to its aggregated prion state, [*URE3*], the activity of Ure2 is impaired. This causes the uptake of poor nitrogen sources in the presence of good ones.¹¹ Rnq1 has no

known function except to influence the rate that other prion proteins such as Sup35 can access their prion conformations.^{17,53-57} This activity manifests itself when Rnq1 is in its prion state, [RNQ⁺]. HET-s is a prion protein, which is unique from the other fungal prions since it is not rich in glutamine and asparagine residues, that exists in the filamentous fungus *Podospora anserina* and is involved in heterokaryon incompatibility.^{58,59} To prevent fusion of fungal strains with different genomes, approaching *P. anserina* colonies undergo trial fusion to test for polymorphisms at a dozen loci. When the HET-s prion protein switches from its soluble non-prion state, [Het-s*], to its aggregated prion state, [Het-s], the insoluble prion protein facilitates programmed cell death for certain incompatible fusions through an unknown mechanism.

An intriguing question is how many more fungal prions are there? Four additional yeast prions have been unambiguously identified recently ([SWT⁺]¹⁵, [MOT3⁺]⁶⁰, [MCA]⁶¹ and [OCT⁺]⁶²), and several other non-Mendelian phenotypes in *S. cerevisiae*,⁶³⁻⁶⁵ *S. pombe*⁶⁶ and *P. anserina*⁶⁷ may be prion-based as well. Many potential prions have been identified by genome-wide analysis of yeast and other organisms for proteins of similar sequence composition to the known yeast prions.^{60,68} Also, the fact that *P. anserina* prion HET-s (and PrP for that matter) are not rich in glutamines and asparagines suggests there may be other such prions.

Fundamentals of the [PSI⁺] prion

Herein we highlight recent studies of Sup35, the most intensely studied yeast prion. Sup35 contains an N-terminal domain rich in uncharged, polar residues (Figure 1). This domain is natively unstructured and governs prion formation. It contains 5.5 imperfect, oligopeptide repeats (PQGGYQQYN) reminiscent of the 5 oligopeptide repeats in PrP (PHGGGWGQ).⁶⁹⁻⁷² The highly charged, middle (M) domain has a strong solubilizing activity and promotes the non-prion state.³⁻⁷ Together these domains (NM) govern Sup35's ability to exist in two states, namely prion (amyloid) and non-prion (soluble) conformers.⁷³ The C-terminal folded domain contains its translation termination activity.³⁻⁷

By ingenious interpretation of diverse genetic experiments, Reed Wickner suggested that Sup35 (and also Ure2) might cause heritable phenotypic change via some sort of “protein only” mechanism.^{11,47} Subsequent genetic, biochemical, and cell biological work by others proved it true and established elegant mechanisms by which it worked.⁷³⁻⁷⁶ Differential sedimentation studies provided critical initial evidence that Sup35, in an aggregated state, enciphers the $[PSI^+]$ phenotype. Sup35 from $[PSI^+]$ yeast lysates localizes to the pelleted fraction, while in $[psi^-]$ lysates it remains in the supernatant.^{13,77} These studies were strengthened by the fact that transient expression of Hsp104, a protein disaggregase, switch cells from the prion to the non-prion state heritably, and when it did so the aggregates of Sup35 disappeared.^{74,75} Expression of GFP-tagged NM allowed monitoring of Sup35 dynamics in living cells.⁷⁶ In $[psi^-]$ yeast the fluorescence was diffuse. But in $[PSI^+]$ yeast it was captured into pre-existing prion foci. Other GFP proteins were not captured. Thus, Sup35 forms aggregates in the prion state that uniquely capture newly made Sup35 protein *in vivo* and convert it to the same aggregated state.⁷⁶

In vitro analysis of the assembly of purified Sup35 and fragments thereof revealed that these proteins have an intrinsic capacity to exist in two distinct states, one of which can template the other to change shape. Purified Sup35 self-assembles into amyloid fibers only after a considerable lag phase *in vitro*.^{78,79} But once these β -sheet rich fibers are formed, even a very small amount of fibers is extremely efficient at “seeding” (i.e., templating) soluble Sup35 to assemble into the same amyloid fiber state. Lysates from $[PSI^+]$ cells have this same seeding capacity, but not lysates from $[psi^-]$ cells.⁷⁷ And mutants which hasten or hindered prion propagation *in vivo* have the same effect on the *in vitro* assembly reactions.⁸⁰ Thus, this self-perpetuating conformational conversion of protein from one functional state to a profoundly different state explained the molecular nature of prion inheritance. This was confirmed when the prion domain of Sup35 was transferred to a completely different protein, the glucocorticoid receptor, and converted that protein to a prion with all of the genetic and biochemical behavior of Sup35.⁸¹

The gold standard for verifying this hypothesis is to start with recombinant protein, assemble it into amyloid fibers *in vitro*, purify and introduce these fibers into the host organism and demonstrate that they induce the prion phenotype. This hypothesis was first confirmed for HET-s,⁴⁴ but was soon demonstrated for Sup35,^{18,19} as well as for other fungal prions.^{45,46,60} In each case, the amyloid conformation was capable of inducing the prion phenotype, while the soluble protein did not do so above background rates of spontaneous prion formation.

Prion Amyloid Structure

Peptide Amyloids

For years the arrangements of amino acids within prion amyloids has been fiercely debated.⁸²⁻⁸⁴ The structures of insoluble amyloids are poorly defined since they are typically refractory to analysis by X-ray diffraction and conventional solution NMR. An important recent breakthrough is that two short overlapping peptides from the extreme N-terminus of Sup35 (residues 7-13 and 8-13) were crystallized and their structures have been studied both by X-ray diffraction⁸⁵ and solid state NMR.⁸⁶ The β -strands are oriented perpendicular to the long axis of the crystals (Figure 2a), as expected for amyloids. The key finding, however, is that two β -sheets bond together in a self-complementing “steric zipper”. Instead of opposing side chains hydrogen bonding with each other, they interdigitate with an extraordinary degree of geometric complementarity that excludes water and stabilizes the structure via van der Waals interactions. The outer faces of the two sheets are highly hydrated and may prevent lateral fiber growth. Short peptides (4-12 residues) from several other amyloid-forming proteins have now been crystallized as well and also show steric zipper structures.⁸⁷ Importantly, interdigitated dry interfaces observed in these structures may explain the remarkable stability of amyloids observed both *in vitro* and *in vivo*. However, no peptide crystals by themselves have biological activity (e.g., induction of $[PSI^+]$ using the protein transformation method^{18,19}). Thus, while they provide a fascinating view of the nature of amyloid interfaces, they are unlikely to be the actual infectious prion interface.

Sup35 amyloids

The structural analysis of amyloids assembled from full-length proteins such as NM is extremely challenging and there is tremendous controversy over the proposed structures.⁸⁸⁻⁹⁰ One prominent model is the in-register parallel β -sheet (Figure 2b).^{89,90} The crux of this model is that each monomer forms an accordion pleat, with each residue in the amyloid core stacked on top of an identical residue from a different molecule, resulting in one molecule per 4.7 Å in the axial direction. Regions not involved in the amyloid core are expected to decorate the surface as loops or pendent chains.

Three principal experiments support the relevance of this structural model. First, mass-per-unit length measurements of amyloids formed from a fragment of NM (residues 1-61) revealed approximately one molecule per 4.7 Å,⁹¹ consistent with the in-register parallel β -sheet model. Second, the sequence of the N domain of Sup35 was scrambled in multiple ways and all were able to induce and propagate prions.⁹² Since self-stacking of identical residues would be unaffected by scrambling (i.e., a residue can stack on itself regardless of the identity of neighboring residues), these results appear to support the parallel β -sheet model. However, the induction frequencies appeared much lower than observed previously for wild-type Sup35 (the wild-type control was not reported). Reduced prion infectivity could be due to the fact that self-stacking is influenced by neighboring residues and parallel β -sheet structures require specific sequences to form efficiently. Alternatively, the parallel β -sheet model could be incorrect.

The third line of evidence that has been cited in support of the parallel in-register model comes from solid state NMR analysis of NM amyloids.⁸⁹ Four amino acids (phenylalanine, tyrosine, leucine and alanine) were separately ¹³C labeled. Using a recoupling method to selectively probe ¹³C-¹³C separation distances, the number of labeled residues within 5 Å was measured to determine which residues are in β -sheets. Since most of these residues do not neighbor identical residues, close proximity between labels must be due to intramolecular or intermolecular structure. For NM amyloids most tyrosine and leucine residues were within 5 Å (>85%), while a smaller fraction of phenylalanine and alanine residues (<65%) were in such close proximity. Shewmaker et al. argue that the close proximity of many residues in both the N and M domains is most consistent with the in-register parallel β -sheet model.⁸⁹

Another prominent model for amyloid structure of NM and other proteins is the β -helix (Figure 2c).^{84,88} Crystal structures of globular β -helical proteins provide some insight into this model.^{84,93} For example, a single rung of a β -helix typically has ~10-20 residues. Moreover, there is a central pore inside the helix that prevents close contact of β -sheets. Therefore, the β -helix model makes two predictions about NM fiber structure: 1) if the amyloid core is long enough to form more than two rungs, then some residues within the core will not be in intermolecular contact and 2) there would not be a 8-10 Å reflection in the X-ray diffraction pattern since β -sheets parallel to the fiber axis are not in close contact.

Results from two studies are consistent with these predictions.^{88,94} First, an exhaustive cysteine scanning mutagenesis study was used to probe NM amyloid structure.⁸⁸ Since NM is devoid of cysteines, 37 single cysteine mutations were introduced throughout its sequence to facilitate site-specific attachment of diverse biochemical probes. Importantly, the cysteine mutations did not influence the rate of amyloid polymerization *in vitro* or the fidelity of prion propagation *in vivo*. To access the degree of solvent accessibility of each cysteine residue, two methods were used. First, monomeric NM cysteine mutants were labeled with fluorescent dyes sensitive to the degree of solvent exposure and then assembled into fibers. As a complementary approach, single cysteine mutants were assembled into fibers and then labeled with fluorescent dyes. For NM monomers labeled with environmentally sensitive fluorophores and then assembled into fibers at 25°C, a contiguous, solvent-shielded amyloid core that encompasses most of the N domain (residues 21-121) was found. The post-assembly labeling results revealed a smaller amyloid core (residues 2-73 were <50% solvent accessible); the difference between these results needs to be resolved. In any case, given the length of this amyloid core (at least 70 amino acids), a β -helix structure would predict more than two rungs. Therefore, it is expected that the central residues in the amyloid core would not be in intermolecular contact, a very different situation than predicted by the in-register parallel β -sheet model.

Indeed, in the same study analysis of the intermolecular proximity of identical residues within NM fibers suggests that not all of the amyloid core is in self-contact.⁸⁸ Single cysteine mutants were

labeled with fluorophores sensitive to inter-dye spacings prior to assembly into amyloid fibers. Two regions within the N domain (~residues 20-40 denoted as the “head” and 90-110 denoted as the “tail”) were in close self-intermolecular contact (4-10 Å), while the intervening region (~residues 40-90) and the M domain were not.

One concern about the pyrene excimer analysis is the use of large fluorescent probes and their potential influence on local amyloid structure. Importantly, an additional independent method using smaller probes in the form of bifunctional, cysteine-reactive cross-linkers also supports the β -helix model.⁸⁸ Cross-linking monomeric cysteine mutants in the head region (~residues 20-40) produced NM dimers that greatly accelerated amyloid formation, while cross-linking in the tail region (~residues 90-110) did not alter the rate of amyloid assembly. However, cross-linking the intervening region (~residues 40-80) inhibited amyloid formation, again suggesting that only a subset of residues in the amyloid core form intermolecular contacts. These and other results appear to be most consistent with the β -helix model;⁸⁸ two regions are in self-intermolecular contact, while the intervening region forms intramolecular contacts.

X-ray diffraction analysis of NM amyloids reveals that the reflection at 8-10 Å may be an artifact of drying the fibers.⁹⁴ For fibers of both N and NM, two reflections (4.7 and 8-10 Å) were observed for dried fibers but only one (4.7 Å) for hydrated fibers. The absence of the equatorial reflection suggests that hydrated NM amyloids are devoid of closely stacked β -sheets in the direction parallel to the fiber axis. This observation led to Kishimoto et al. to first propose the β -helix model for NM amyloid structure.⁹⁴ However, this study is controversial since the diffraction pattern is much weaker for the hydrated samples and may limit detection of the equatorial reflection.⁹⁵

To reconcile these dissimilar models of NM prion structure (i.e., in-register parallel β -sheet vs β -helix), it is essential to employ independent methods of amyloid structural analysis. Indeed, a recent heroic study of NM fiber structure using hydrogen/deuterium (H/D) addresses some discrepancies between these models.⁹⁶ Mature NM amyloids were exposed to deuterium, dissolved in DMSO and the

degree of H/D exchange was probed by solution NMR. Like solid state NMR, this approach is time consuming and technologically challenging given the highly degenerate sequence of NM. While no specific structural model is proposed in this study, the NM amyloid core that formed at 37°C encompassed residues ~5-70. This is remarkably similar to the amyloid core (residues 2-73) identified by the much simpler cysteine accessibility studies for NM fibers that form at 25°C (fibers formed at 25 and 37°C have similar thermal stabilities¹⁹ and apparently similar structures).⁸⁸ That fact that these two disparate methods give such similar results is a significant accomplishment in the controversial field of amyloid structural analysis. Moreover, these results suggest that analysis of cysteine accessibility in prion amyloids is a powerful and straight-forward approach for identifying which residues are within the amyloid core. Finally, both results differ significantly from the residues predicted to be structured in β -sheets by solid state NMR results (most of residues 1-123 and a portion of residues 124-253).⁸⁹ The lack of agreement may be due to the inability of solid-state NMR to discriminate between β -sheets with different stabilities, while labeling methods (H/D exchange and alkylation of cysteines) may be capable of such discrimination since highly stable β -sheets are labeled more slowly than less stable β -sheets.

How can controversies regarding different Sup35 structural models be resolved? Site- and segment-specific labeling methods appear to hold the key. Until structural properties of individual amino acids or small segments of amino acids within prion amyloids are studied in a systematic manner, it is unlikely that a single structural model will emerge from this controversy. For solid state NMR studies, single positions within proteins could be ^{13}C or ^{15}N labeled by introducing mutations encoding residues in the Sup35 prion sequence not naturally present (e.g., tryptophan) and using expression media with only these amino acids isotopically labeled. Moreover, ^{13}C or ^{15}N labeled peptide segments could be introduced into otherwise unlabelled Sup35 protein using inteins or other ligation methods.^{97,98} Finally, use of side-chain specific reagents that covalently modify proteins,⁹⁹ even reagents other than cysteine-reactive molecules,¹⁰⁰ coupled with NMR or mass spectrometry stand to make important contributions for resolving these controversies.

Prion Strains

One of the most perplexing aspects of prions is their ability to form different structural strains.¹⁷⁻²³ Prion proteins have long been speculated to access not only one infectious amyloid conformation, but a suite of related yet distinct, self-perpetuating conformations, that encode different biological phenotypes.¹⁰¹ Recently this was demonstrated unequivocally by transforming yeast with NM amyloids with different physical properties and demonstrating that they produce distinct phenotypes.^{18,19} An enabling breakthrough in this study was that different NM amyloid conformations could be formed simply by assembling fibers at different temperatures (e.g., 4 vs. 25°C).¹⁹ Tanaka et al. demonstrated that there are gross structural differences between the two populations of fibers by measuring differences in their stabilities (e.g., fibers formed at 4°C melt at lower temperatures than those formed at 25°C).¹⁹ When amyloids formed at 4°C were transformed into yeast, they generally produced a relatively high degree of read-through of stop codons and, hence, a strong [*PSI*⁺] phenotype. Conversely, transformation of yeast with amyloids formed at 25°C encode a lower degree of read-through and a weak [*PSI*⁺] phenotype.

This elegant protocol to form different prion strains has led to several studies of their structural differences.^{19,40,88,96} Krishnan and Lindquist found two important differences in the structures of NM amyloids formed at 4°C and 25°C.⁸⁸ First, single cysteine NM mutants labeled with dyes whose fluorescence depends on solvent exposure prior to assembly revealed that there are many fewer residues in the amyloid core for fibers formed at 4°C (~residues 31-86) than for those formed at 25°C (~residues 21-121). The smaller amyloid core for the 4°C fibers is consistent with their lower melting temperature and higher propensity to be fragmented *in vitro* relative to 25°C fibers.^{19,102} Second, the location of one of the intermolecular contact regions is strongly shifted while the other is somewhat shifted.⁸⁸ For both amyloid conformations, residues ~20-40 (head region) form an intermolecular contact. An additional contact is seen at the extreme N-terminus for 25°C fibers. However, the second intermolecular contact (tail region) encompasses residues ~80-100 for 4°C fibers and ~90-110 for 25°C fibers.

Are differences in the intermolecular contacts sufficient to determine the formation of unique prion strains? If so, cross-linking NM proteins in the head and tail regions should bias formation of different strain conformations in a manner independent of the temperature at which they nucleate. Indeed, cross-linking NM proteins in the head region yields dimers that form strong prion strains regardless of the nucleation temperature (4 or 25°C).⁸⁸ Conversely, cross-linking NM proteins in the tail region yields dimers that form weak strains regardless of the nucleation temperature.⁸⁸ The fact that it is the nature of the intermolecular contact that determines the nature of the strain explains how these properties can be self-perpetuating, because strains are propagated from the templating surface. Similar analysis for other prions will determine the generality of these exciting insights into prion strain nucleation.

Encouragingly, several of these structural insights have been confirmed by an independent method of amyloid structural analysis, namely hydrogen/deuterium (H/D) exchange coupled with solution NMR.⁹⁶ Residues ~4-40 were most protected for the 4°C fibers, while residues ~4-70 were most protected for the 37°C fibers. These results are qualitatively similar to those obtained by labeling NM cysteine mutants with acrylodan prior to assembly.⁸⁸

An extensive mutagenesis study recently strengthened the idea that prion strain variation is due to differences in the size of the amyloid core.¹⁰³ King and coworkers systematically introduced mutations (proline substitutions or glycine insertions) that destabilize amyloid structures throughout the prion domain of Sup35. Interestingly, they found that mutations in largely continuous peptide segments prevented prion propagation *in vivo*, and that three prion strain variants displayed unique stretches of amino acids (ranging from as small as residues 7-21 to as large as residues 5-55) that could not be mutated without causing loss of the prion state.

A common theme of the Sup35 prion strains studied to date is that they display relatively large structural variations (e.g., regions shielded by solvent differ by more than 10 residues). However, Eisenberg and coworkers recently illuminated more subtle structural changes (that do not require significant changes in solvent exposure) that may also contribute to the unique biological properties of

strain variants.⁸⁷ Through careful analysis of steric zipper structures of several short peptide fragments (4-12 residues) from different amyloid-forming proteins (e.g., Su35, PrP, and A β), several arrangements of peptides in amyloid-like conformations were identified. Eight different steric zipper structures are expected based on the possible permutations of the orientation of peptides within each β -sheet (parallel or anti-parallel) and how peptides in different β -sheets are oriented relative to each other. Interestingly, not only did Eisenberg and co-workers experimentally confirm that at least five out of the eight steric zippers can form, but also that individual peptide fragments from Sup35 (⁸NNQQ¹¹) and other amyloid-forming proteins also can form multiple types of steric zippers (Figure 4). Unfortunately, the large structural differences observed for different Sup35 prion strains^{88,96} cannot be mapped on these small peptides. However, the diversity of the structures provides a fascinating glimpse into the nature and variety of prion amyloid packings and polymorphic structures. Analysis of the biological role of steric zippers in the context of larger polypeptides with known prion activities is an exciting area of future research.

Prion Species Barriers

Elucidating how prions establish and overcome species barriers is a key pursuit in the field of prion biology. An important molecular determinant of species barriers is the primary sequence of prions. For example, this was illuminated through the study of Sup35 prions from the yeast species *S. cerevisiae* (Sc), *C. albicans* (Ca) and *P. methanolica* (Pm).³¹ Each protein efficiently formed self-perpetuating prions when overexpressed, but none cross-catalyzed conversion of the same proteins from the other species. The species barrier between the NM domains of *S. cerevisiae* (ScNM) and *C. albicans* (CaNM) was confirmed *in vitro*; amyloid fibers of ScNM could template polymerization of ScNM, but not for CaNM, and vice versa.³¹ This and other studies^{29-35,39} established the utility of studying prion species barriers in yeast.

Surprisingly, much can be learned about how prions establish and overcome species barriers using libraries of immobilized, short peptide fragments.⁴¹ Overlapping peptides (20mers) that encompass the entire sequence of ScNM and CaNM were arrayed on glass slides, and used to interrogate the role of

both prion sequence and structural variation on the ability of prions to overcome species barriers. Fluorescently-labeled ScNM and CaNM proteins each bound to a small set of their own peptides (ScNM residues 9-39 and CaNM residues 59-86; Figure 5). Importantly, they did not cross-react with each other.⁴¹ The amino-acid sequences bound by each protein were named “recognition elements”. Closer inspection of this binding revealed that each prion protein nucleated into amyloids upon binding to peptides in their own recognition elements. Moreover, the specificity of binding of each prion protein suggests that the species barrier is enciphered by small elements of primary sequence. Indeed, a Sc/Ca NM chimeric prion capable of traversing this species barrier bound to peptides from both species, unlike either ScNM or CaNM proteins.⁴¹

Prion species barriers are also highly dependent on the conformational diversity of prion strains.^{4,8,9,24,26,27,29,37,40} It is likely that mammalian prions were transmitted from cattle to humans through a specific, highly infectious prion conformation.^{8,9,24,26,27,37} This fascinating interdependence has recently been interrogated in yeast.^{29,30,40} The Sc/Ca NM chimera can form different amyloid conformations with unique propensities to cross species barriers by simple manipulations such as altering the temperature at which they assemble.^{29,30} One conformation of the chimeric prion is specific for infecting *S. cerevisiae*, while the other conformation is specific for infecting *C. albicans*. Using peptide microarrays the molecular origins of this behavior were elucidated. The chimeric prion bound selectively to peptides in the ScNM sequence at 15°C. In contrast, it bound selectively to CaNM peptides at 37°C. These and related results show remarkable correspondence to the species-specific seeding activities of the two chimeric strains.²⁹ Selective binding of the chimera to CaNM peptides at 37°C reflects the assembly of chimeric amyloids that selectively infect *C. albicans*. And the selective binding of the chimeric prion to ScNM peptides at 4°C reflects the assembly of prion amyloids that selectively infect *S. cerevisiae*. These results indicate it is nucleation at the recognition elements that regulates formation of an amyloid conformation that will perpetuate seeding specificity for the same recognition sequence.

Prion Nucleation and Oligomerization

As discussed above, prion nucleation is the basis for multiple facets of prion strains and species barriers. An important aspect of nucleation is the context, namely the oligomerization state, during which conformational change occurs. Serio et al. first identified that NM forms spherical, structurally-fluid oligomeric structures during amyloid assembly.¹⁰⁴ These oligomers were observed initially by AFM and TEM,¹⁰⁴ and later by dynamic light scattering.¹⁰⁵ Several different lines of evidence established that these oligomers were on pathway for amyloid formation (for a conflicting report, see ref¹⁰⁶).^{104,107} Nucleation through the formation of specific intermolecular contacts within molten oligomers provided a completely different explanation for the lag phase in the assembly of this polypeptide than previously established for the assembly of actin and tubulin,¹⁰⁸ and solved the Levinthal paradox for protein folding^{104,109} of amyloidogenic proteins. This protein folding paradox states that finding the global energy minimum and finding it quickly are mutually exclusive. For a large unstructured protein like NM, it appears that folding in the context of oligomers leads to acceleration of proper amyloid folding pathways while limiting sampling of other pathways, yielding specific amyloid conformations on biologically relevant time scales.

Importantly, since this initial report, other prions and many other amyloid-forming proteins have been found to nucleate via very similar oligomeric intermediates,^{13,46,110-115} and these intermediates are widely posited to be the key toxic species in numerous protein misfolding diseases.^{111,112,116,117} Remarkably, a conformationally-specific antibody initially developed to recognize oligomeric intermediates to the A β peptide recognizes NM oligomers,^{112,118} as well as oligomeric intermediates formed by several other proteins.¹¹² This antibody inhibits amyloid formation of both NM and full-length Sup35,¹⁰⁷ confirming that NM oligomers are an obligate structural intermediate in the nucleation of infectious prion conformers. Nevertheless, very little is known about these structures, and elucidating their dynamic structural evolution during nucleation is an important pursuit in coming years. Single molecule approaches for studying protein nucleation, such as those used to study NM¹¹⁹ and polyQ¹²⁰, are well suited for such studies.

Conclusions and Perspectives

The biochemical analysis of yeast prions has produced many important findings that have shed light on their enigmatic properties. However, much remains unknown about these captivating proteins. Understanding how prions nucleate into infectious amyloid conformers is critical to unlock unanswered questions about prion strains and species barriers. Advances in amyloid structural analysis should enable new insights into the molecular basis of prion strains and better definition of the extent of structural differences between different prion conformers. In turn, these structural insights will aid in further elucidating the molecular basis of how different prion strains have unique capacities to overcome prion species barriers. This analysis is not only relevant to prion biology, but also to the pathogenic role of pre-amyloid (oligomeric) structures in many neurodegenerative diseases, where conversion to amyloid forms, with diverse strain properties, may be neuroprotective.¹²¹ Amyloid formation has also recently been shown to be the basis of melanin production in mammals,¹²² the basis of biofilm formation in microorganisms,¹²³ and appears to play a role in long term memory in neurons.¹⁴ Finally, the recent discovery of several new prions,^{15,60-62} some of which confer strong beneficial traits in particular environments,⁶⁰ and the realization that proteotoxic stress increases prion switching rates¹²⁴ support the exciting hypothesis that prion amyloids serve as “bet-hedging” strategies, vastly increasing heritable phenotypic diversity.⁶⁰ A whole new world of amyloid-based biology is unfolding before our eyes. Heroic efforts to solve the challenging problems these proteins present in the realm of protein folding will be well worth the effort.

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Figure Captions

Figure 1. Molecular basis of $[PSI^+]$ prion propagation. Isogenic *Saccharomyces cerevisiae* in the (A) $[psi^-]$ and (B) $[PSI^+]$ states. The protein determinant of $[PSI^+]$, Sup35, is (C) soluble and complexed to Sup45 in the $[psi^-]$ state and (D) insoluble and inactive in the $[PSI^+]$ state. The inactivation of Sup35 causes read-through of stop codons and large phenotypic changes, some of which are beneficial.⁴⁹⁻⁵¹ (E) Molecular architecture of Sup35. (F) Primary sequence of the prion (N) domain of Sup35.

Figure 2. Amyloid structures of prion peptides and proteins. (a) Crystal structure of ⁷GNNQQNY¹³, a 7-mer peptide from the N-terminus of Sup35.⁸⁵ The crystal structure reveals a high degree of geometric complementarity between opposing strands, which leads to exclusion of water at this interface and explains the stability of these amyloids. (b) In-register, parallel β -sheet model of NM amyloid structure based on solid-state NMR results.⁸⁹ This model proposes that most of the residues in the N domain and some residues in the M domain self-stack (such as indicated residue, Y101). (c) β -helix model of NM amyloid structure.⁸⁸ This model proposes that two amino acid segments in the N domain are in intermolecular contact, while the intervening region is intramolecular contact.

Figure 3. Steric zipper structural variants of a Sup35 peptide fragment. Crystal structures of the Sup35 peptide ⁸NNQQ¹¹ in two of eight possible steric zipper structures.⁸⁷ (a) Parallel β -sheet steric zipper structure where the faces of identical peptides in different β -sheets face each other and are both oriented upward. (b) Similar β -sheet steric zipper structure where the opposite faces of peptides in different β -sheets face each other and the orientation of peptides in the second β -sheet is down relative to the upward orientation of peptides in the first β -sheet.

Figure 4. Species-specific infectivities of prion strains. A chimeric Sup35 prion, composed of N-terminal and middle domains (collectively referred to as NM) of *S. cerevisiae* (Sc) and *C. albicans* (Ca)

Sup35, nucleates into two different prion amyloid conformations at different temperatures with species-specific infectious properties. Peptide microarray analysis reveal that this prion has two small regions of primary sequence (recognition sequences) that regulate its nucleation behavior, one from Sc domain of this prion and the other from the Ca domain.⁴¹ Low temperatures favor nucleation from the Sc recognition sequence and generate an amyloid conformation specific for templating Sc Sup35 monomers.^{29,30} High temperatures favor nucleation from the Ca recognition element and generate an amyloid conformation with the opposite templating specificity.

Definition Box 1

Prion protein	Any polypeptide that, in addition to its normal conformation (which is typically soluble), can access at least one conformation (which is typically β -sheet rich and insoluble) that is self-perpetuating and infectious
Amyloid	A highly stable structure composed of many protein monomers arranged into β -sheet rich fibrils such that the β -strands from different monomers stack perpendicular to the fibril axis
Prion strains (variants)	Distinct prion diseases or phenotypes that are caused by unique β -sheet rich conformations of infectious prion proteins with identical amino acid sequence
Prion species barriers	A phrase describing the inefficient transmission of infectious prions between different species
Templating	The process by which infectious prions catalyze the conformational change of proteins (that are typically identical in amino acid sequence) from their soluble, non-prion conformation to their insoluble, prion conformation

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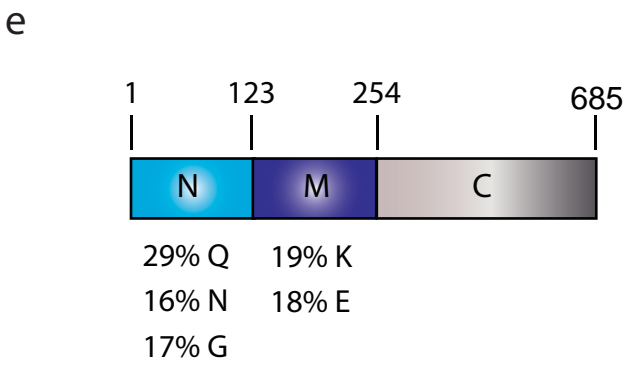
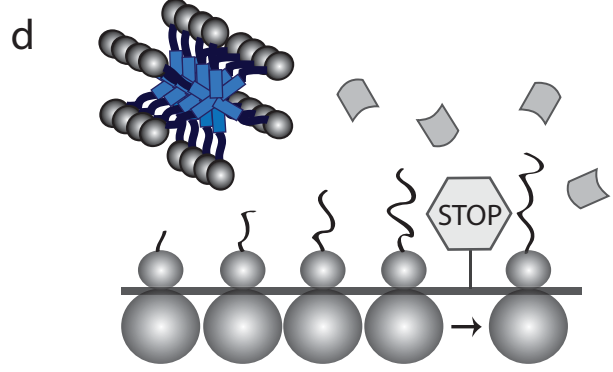
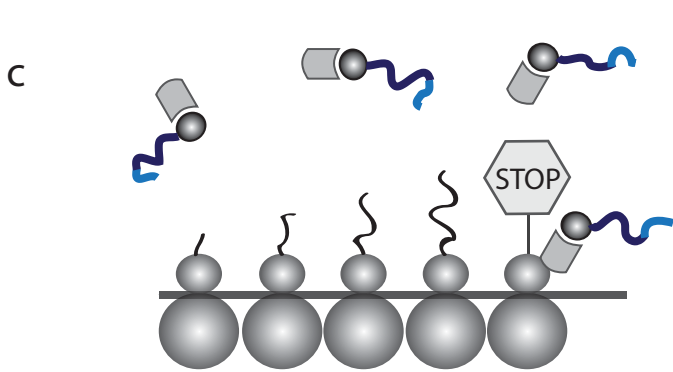
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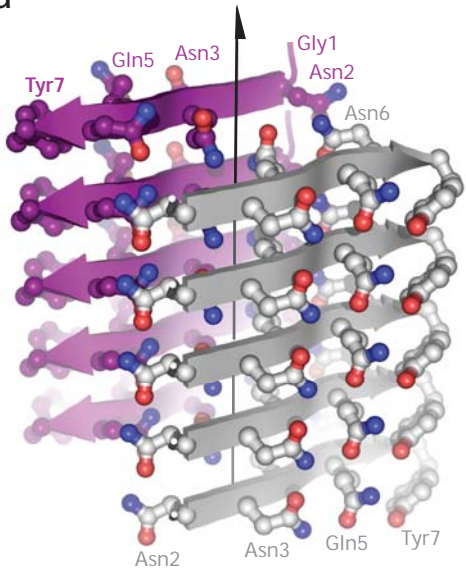
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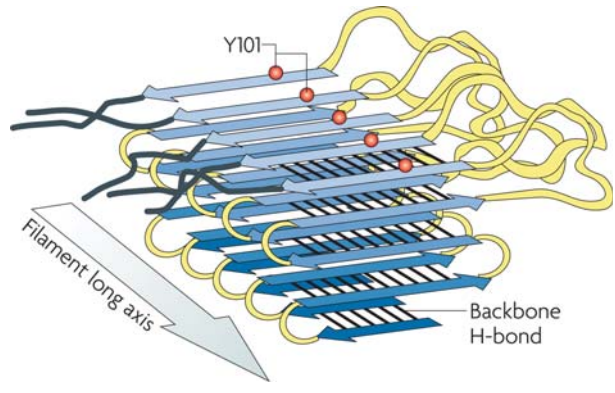
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Figure 1. Tessier & Lindquist

a



b



c



Figure 2. Tessier & Lindquist

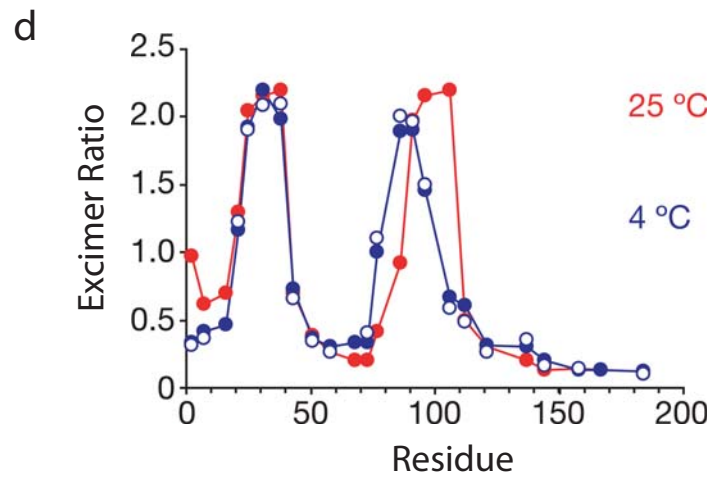
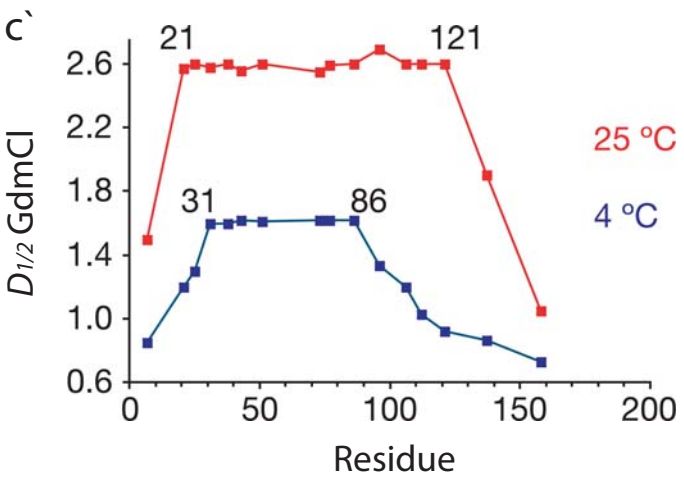
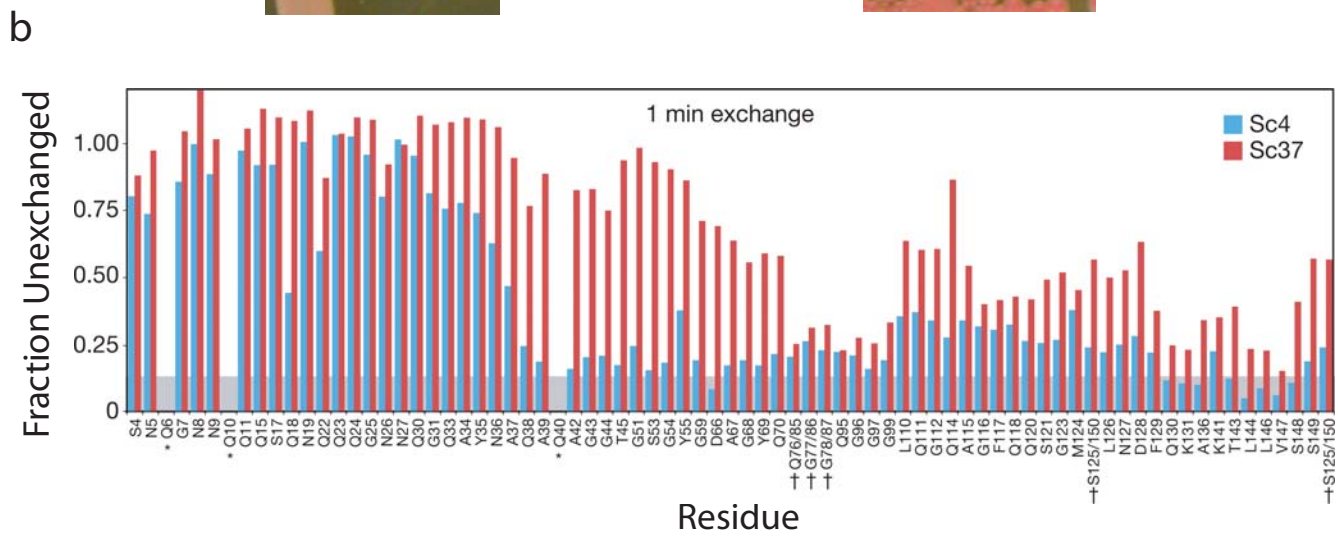
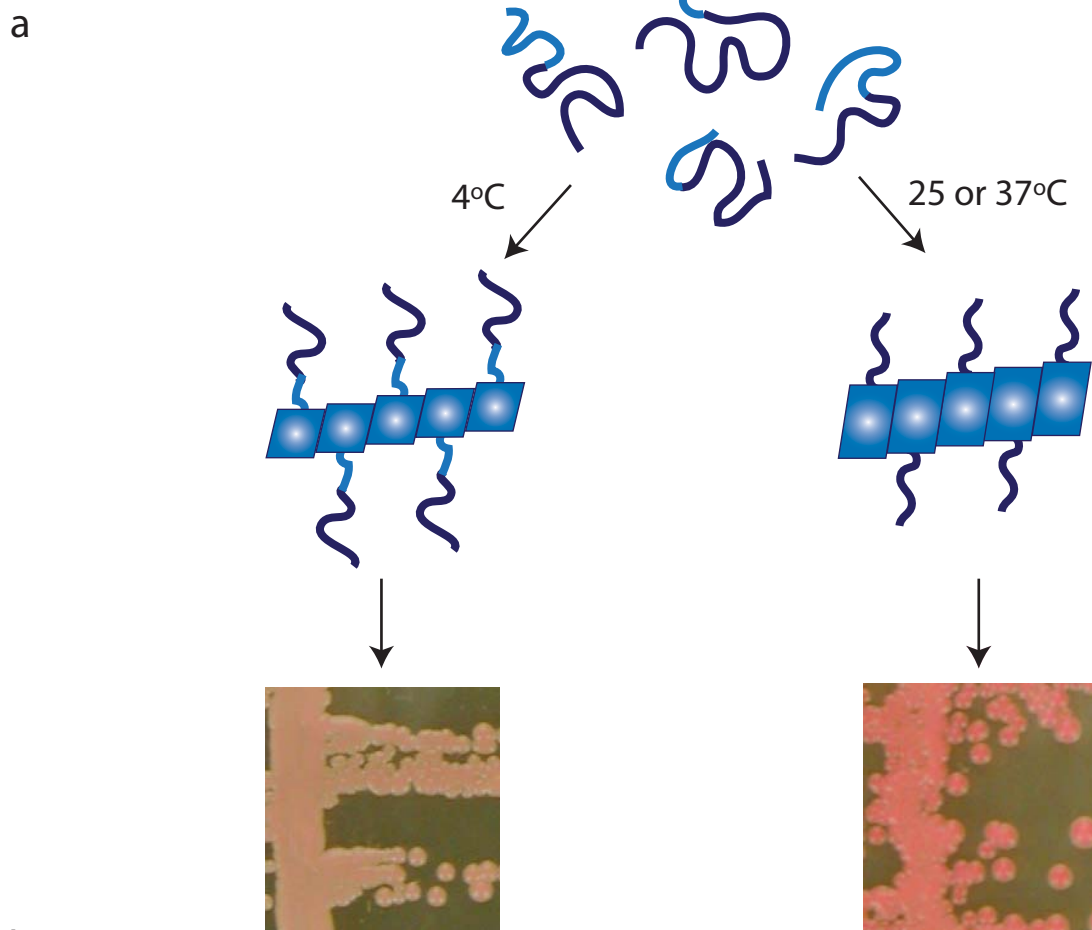


Figure 3. Tessier & Lindquist

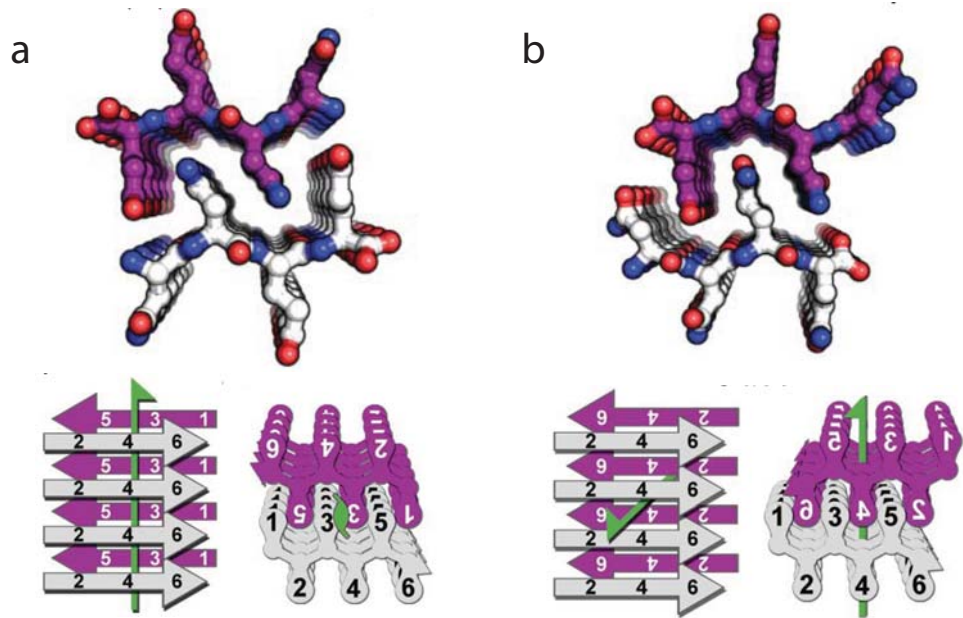


Figure 4. Tessier & Lindquist

