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Unraveling infectious structures, strain variants and species barriers for the yeast prion [*PSI*$^+$]

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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 show several remarkable biological activities, including the ability to form multiple infectious prion conformations, also known as strains or variants, encoding unique biological phenotypes, and to establish and overcome prion species (transmission) barriers. In this Perspective, 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 such biological activities. We also discuss several aspects of prion conformational change and structure that remain either unknown or controversial, and we propose approaches to accelerate the understanding of these enigmatic, infectious conformers.

The 'misfolding' and assembly of proteins into β-sheet–rich amyloid fibers is important in both disease[1] and normal biological function[2,3]. Although many proteins form amyloid fibers *in vitro* (see **Table 1** for definitions), 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 the protein in question was identified as 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 alternative conformation appears in the brain—via 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].

Despite a wealth of evidence, 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 owing 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 the discovery of a host of new prions with diverse functions in yeast (for example, see refs. 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 crucially important feature shared by mammalian and fungal prions is the species barrier[9,24,25,29–38]. The aforementioned prion strains show 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 show strong species barriers that can be crossed, but with difficulty[29–32,34,35,39–41]. 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 in *Saccharomyces cerevisiae* and HET-s in *Podospora anserina*[3–7]. Sup35 is a protein involved in recognition of stop codons during protein

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Table 1 Glossary

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 perpendicularly 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.

synthesis (**Fig. 1**). Conversion of Sup35 from its soluble non-prion state, [*psi*$^-$], to its aggregated prion state, [*PSI*$^+$], causes reduced termination activity[12,47,48] (**Fig. 1a–c**). The resulting increase in read-through of stop codons reveals complex phenotypes that can be beneficial in some cases[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, causing the uptake of poor nitrogen sources in the presence of good ones[11]. Rnq1 has no known function besides influencing the rate at which 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 unique among fungal prions because it contains few glutamine and asparagine residues. HET-s 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. Upon switching 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 ([*SWI*$^+$][15], [*MOT3*$^+$][16], [*MCA*][60] and [*OCT*$^+$][61]), and several other non-Mendelian phenotypes in *S. cerevisiae*[62–64], *Schizosaccharomyces pombe*[65] and *P. anserine*[66] 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[16,67]. In addition, the fact that the *P. anserina* prion HET-s (and PrP for that matter) is not rich in glutamines and asparagines suggests that there may be other such prions.

Fundamentals of the [*PSI*$^+$] prion

Herein we highlight recent studies of Sup35, the best-studied yeast prion (**Fig. 1**). Sup35 contains an N-terminal domain that is rich in uncharged, polar residues, with 5.5 imperfect repeats (PQGGYQQYN) reminiscent of the repeats in PrP (PHGGGWGQ)[68–71] (**Fig. 1d**). The N domain is natively unstructured and governs prion formation, whereas 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[72]. 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 a protein-only mechanism[11,47]. Subsequent genetic, biochemical and cell biological work by others proved this to be true and established the molecular mechanisms involved[48,72–74]. Differential sedimentation studies initially showed that Sup35 from [*PSI*$^+$] yeast lysates localizes to the pelleted fraction, whereas in [*psi*$^-$] lysates Sup35 remains in the supernatant[13,75], indicating that Sup35, in an aggregated state, enciphers the [*PSI*$^+$] phenotype. This concept was strengthened by the observation that transient expression of Hsp104, a protein disaggregase, switches cells from the prion to the non-prion state heritably with the concomitant disappearance of Sup35 aggregates[48,73]. Expression of green fluorescent protein (GFP)-tagged NM allowed monitoring of Sup35 dynamics in living cells: the fluorescence was diffuse in [*psi*$^-$] cells, but the fusion protein was captured into pre-existing prion foci in [*PSI*$^+$] cells, whereas other GFP proteins were not captured[74]. 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.

In vitro analysis of purified Sup35 and fragments thereof revealed that these polypeptides have an intrinsic capacity to exist in two distinct states, one of which can template the other to change conformation. Purified Sup35 self-assembles into amyloid fibers only after a considerable lag phase *in vitro*[72,76,77]. But once these β-sheet rich fibers are formed, even a very small amount of fibers is extremely efficient at 'seeding' (that is, templating) soluble Sup35 to assemble into the same amyloid fiber state. Lysates from [*PSI*$^+$] cells, but not from [*psi*$^-$] cells, have this same seeding capacity[75]. And Sup35 mutants that hasten or hinder prion propagation *in vivo* have the same effect on the *in vitro* assembly reactions[78]. 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

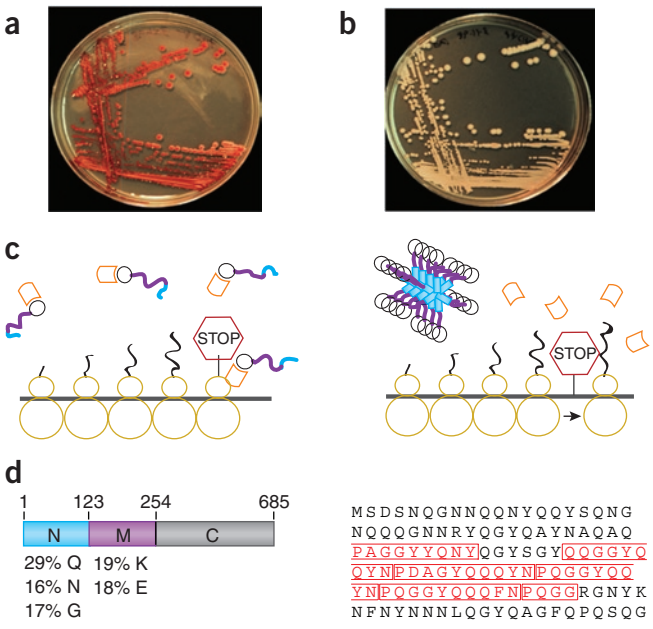

Figure 1 Molecular basis of [*PSI*$^+$] prion propagation. (**a**,**b**) Isogenic *S. cerevisiae* in the [*psi*$^-$] (**a**) and [*PSI*$^+$] (**b**) states. (**c**) The protein determinant of [*PSI*$^+$], Sup35, is soluble and complexed to Sup45 (orange) in the [*psi*$^-$] state (left) or insoluble and inactive in the [*PSI*$^+$] state (right). The inactivation of Sup35 causes read-through of stop codons and large phenotypic changes, some of which can be beneficial[49–51]. (**d**) Domain architecture of Sup35 (left), with the primary sequence (oligopeptide repeats highlighted in red) of the prion (N) domain of Sup35 (right).

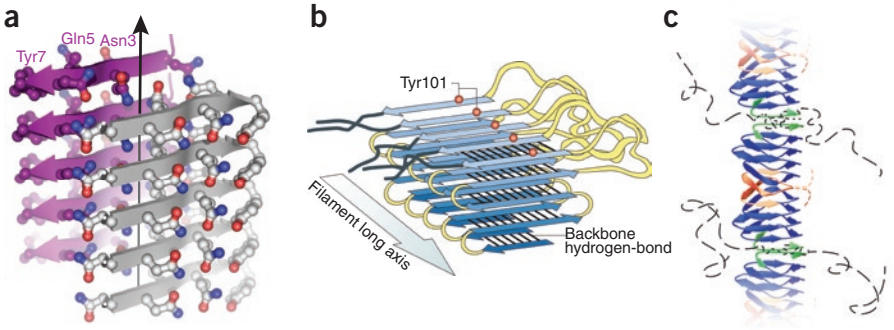

Figure 2 Amyloid structures of prion peptides and proteins. (**a**) Crystal structure of 7-GNNQQNY-13, a 7-mer peptide from the N terminus of Sup35 (reproduced from *Nature*, ref. 83). The crystal structure reveals a high degree of geometic complementarity between opposing strands, which leads to exclusion 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 (reproduced from *Nature*, ref. 87). This model proposes that most of the residues in the N domain and some residues in the M domain self-stack (such as the indicated residue, Tyr101). (**c**) β-helix model of NM amyloid structure[86]. This model proposes that two amino acid segments in the N domain are in intermolecular contact, whereas the intervening region makes intramolecular contacts. The head-to-head (red) and tail-to-tail (green) intermolecular contacts are expected to be in an antiparallel orientation (reproduced from *Nature*, ref. 86).

receptor, and converted that protein to a prion, with all of the genetic and biochemical behavior of Sup35 (ref. 79).

The gold standard for verifying the prion 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 (refs. 18,19), as well as for other fungal prions[16,45,46]. In each case, the amyloid conformation was capable of inducing the prion phenotype, whereas 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[80–82]. The structures of insoluble amyloids are poorly defined, because they are typically refractory to analysis by X-ray diffraction and conventional solution NMR. An important recent breakthrough was the determination of the structure of two short overlapping peptides from the extreme N terminus of Sup35 (residues 7–13 and 8–13) both by X-ray diffraction[83] and solid-state NMR[84]. The β-strands are oriented perpendicularly to the long axis of the crystals (**Fig. 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 and also show steric zipper structures[85]. Importantly, the interdigitated dry interfaces observed in these structures may explain the remarkable stability of amyloids observed both *in vitro* and *in vivo*. However, the peptide crystals by themselves have no known biological activity (for example, induction of [*PSI*$^+$] using the protein-transformation method[18,19]). Thus, although they provide a fascinating view of the nature of amyloid interfaces, they are unlikely to represent the actual infectious prion interface.

Sup35 amyloids. The structural analysis of amyloids assembled from larger fragments such as NM or full-length Sup35 is extremely challenging, and there is tremendous controversy over the proposed structures[86–88]. One prominent model is the in-register parallel β-sheet[87,88] (**Fig. 2b**). The crux of this model is that each monomer forms an accordion pleat, with each residue stacked on top of an identical residue from another monomer, 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 pendant chains.

Three main lines of evidence support the relevance of the in-register parallel β-sheet 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[89], consistent with the model. Second, the sequence of the N domain was scrambled in multiple ways, and all variants were able to induce and propagate prions[90]. Self-stacking of identical residues would be unaffected by scrambling, as a residue can stack on itself regardless of the identity of neighboring residues. However, the induction frequencies were much lower than observed previously for wild-type Sup35 (the wild-type control was not reported), which 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.

The third line of evidence comes from solid-state NMR analysis of NM amyloids[87]. Four amino acids (phenylalanine, tyrosine, leucine and alanine) were separately ^{13}C labeled, and the number of labeled residues within 5 Å was measured using a recoupling method to selectively probe ^{13}C-^{13}C distances. As 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 (>85%) were within 5 Å, with a smaller fraction of phenylalanine and alanine residues (<65%) within such close proximity, which Shewmaker *et al.* argue to be consistent with the in-register parallel β-sheet model[87].

Another prominent model for amyloid structure of NM and other proteins is the β-helix[82,86] (**Fig. 2c**). Crystal structures of globular β-helical proteins provide some insight into this model[82,91]: a single rung of a β-helix typically has approximately 10–20 residues, and a central pore inside the helix prevents close contact of β-sheets. Therefore, the β-helix model makes two predictions about NM fiber structure: (i) if the amyloid core is long enough to form more than two rungs, then some residues within the core will not make intermolecular contacts; (ii) there would not be an 8–10-Å reflection in the X-ray diffraction pattern, because β-sheets parallel to the fiber axis are not in close contact.

Two studies present results consistent with these predictions[86,92]. First, an extensive cysteine-mutagenesis study was used to probe NM amyloid structure (wild-type NM is devoid of cysteine)[86]. In this work, 37 single-cysteine mutations were introduced throughout the NM sequence; importantly, these mutations did not influence the rate of amyloid polymerization *in vitro* or the fidelity of prion propagation *in vivo*. The degree of solvent accessibility of each cysteine residue was assessed by labeling the mutant monomers with fluorescent dyes sensitive to solvent exposure and then assembling them into fibers or, in a complementary approach, by first assembling the mutants into fibers and then labeling the fibers with fluorescent dyes. Using the first approach, a contiguous,

solvent-shielded amyloid core encompassing most of the N domain (residues 21–121) was found. The post-assembly labeling results revealed a smaller amyloid core, as 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, the central residues in the amyloid core would not be in intermolecular contact, a very different situation than that predicted by the in-register parallel β-sheet model. Indeed, when single-cysteine mutants were labeled with fluorophores sensitive to inter-dye spacings before assembly into amyloid fibers, two regions within the N domain (approximately residues 20–40 or the 'head', and residued 90–110 or the 'tail') were in close self-intermolecular contact (4–10 Å), whereas residues in the intervening region (approximately residues 40–80) and the M domain were not.

An independent method, using smaller, cysteine-reactive cross-linker probes to minimize their potential influence on local amyloid structure, also supports the β-helix model[86]. Cross-linking monomeric cysteine mutants in and near the head region produced NM dimers that greatly accelerated amyloid formation, whereas cross-linking in the tail region did not alter the rate of amyloid assembly. However, cross-linking the intervening region inhibited amyloid formation, again suggesting that only a subset of residues in the amyloid core form intermolecular contacts. These and other results seem to be most consistent with the β-helix model[86]: the head and tail regions are in self-intermolecular contact, whereas 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[92]. For fibers of both N and NM, two reflections (4.7 Å 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 Kishimoto *et al.* to first propose the β-helix model for NM amyloid structure[92]. However, this study is controversial, because the diffraction pattern is much weaker for the hydrated samples and may limit detection of the equatorial reflection[93].

To reconcile these dissimilar models of NM prion structure (that is, in-register parallel β-sheet versus β-helix), it is essential to use independent methods of amyloid structural analysis. Indeed, a recent heroic study of NM fiber structure addresses some discrepancies between these models using hydrogen/deuterium (H/D) exchange: NM amyloids were exposed to deuterium and dissolved in DMSO, and the degree of H/D exchange was assessed by solution NMR[94]. As for solid-state NMR, this approach is time consuming and technologically challenging given the highly degenerate sequence of NM. Although no specific structural model was proposed in this study, the NM amyloid core formed at 37 °C encompassed approximately residues 5–70, which is remarkably similar to the core (residues 2–73) identified by cysteine-accessibility studies for NM fibers formed at 25 °C (fibers formed at 25 °C or 37 °C have similar thermal stabilities[19] and apparently similar structures)[86]. Both sets of results differ 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)[87]. The lack of agreement may be due to the inability of solid-state NMR to discriminate between β-sheets with different stabilities; in contrast, methods such as H/D exchange and alkylation of cysteines are capable of resolving highly stable β-sheets (from less stable ones) because they are labeled more slowly.

Other approaches will have an important role in resolving the controversies regarding different Sup35 structural models, with site- and segment-specific labeling methods seeming 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 that are not naturally present in the Sup35 prion sequence (for example, tryptophan). Moreover, ^{13}C- or ^{15}N-labeled peptide segments could be introduced into otherwise unlabeled Sup35 protein using inteins or other ligation methods[95,96]. Finally, use of side chain–specific reagents that covalently modify proteins[97], or reagents other than cysteine-reactive molecules[98], coupled with NMR or MS 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 (see definitions in **Table 1**)[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[99]. Recently, this was demonstrated unequivocally by transforming yeast with NM amyloids with different physical properties that produced distinct phenotypes[18,19]. An enabling breakthrough for these studies was that different NM amyloid conformations could be formed simply by assembling fibers at different temperatures[19] (for example, 4 °C versus 25 °C; **Fig. 3**). There are gross structural differences between the two populations of fibers, as indicated by their different thermal stabilities[19] (for example, fibers formed at 4 °C melt at lower temperatures than those formed at 25 °C). 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 led to a lower degree of read-through and a weak [*PSI*$^+$] phenotype (**Fig. 3a**).

This elegant protocol to form different prion strains has led to several studies of their structural differences[19,40,86,94]. First, single-cysteine NM mutants labeled with fluorescent dyes sensitive to solvent exposure before assembly revealed that there are far fewer residues in the amyloid core for fibers formed at 4 °C (approximately residues 31–86) than for those formed at 25 °C (approximately residues 21–121)[86] (**Fig. 3b**). The smaller amyloid core for the 4 °C fibers is consistent with their lower melting temperature and higher propensity to be fragmented *in vitro* and *in vivo* compared to the 25 °C fibers[19,100]. Such structural insights have been supported by an independent approach, namely, H/D exchange coupled with solution NMR[94] (**Fig. 3d**): residues 4–40 were most protected for the 4 °C fibers, whereas residues 4–70 were most protected for the 37 °C fibers. Second, the location of the head intermolecular contact region (approximately residues 20–40) is somewhat shifted, with an additional contact seen at the extreme N terminus for 25 °C fibers. Moreover, the second intermolecular contact (tail) encompasses approximately residues 80–100 for 4 °C fibers and approximately residues 90–110 for 25 °C fibers (**Fig. 3c**). The seeming paradox that prions strains with stronger, more stable phenotypes *in vivo* correspond to fibers with shorter, less stable amyloid cores *in vitro* is readily explained[100]. Easier fragmentation of the smaller amyloid cores yields more fiber ends for rapid templating[101] and facilities partitioning of prion seeds to daughter cells.

If the differences in the intermolecular contacts are sufficient to determine the formation of unique prion strains, cross-linking NM in the head or tail regions should bias formation of different strain conformations in a temperature-independent manner. Indeed, cross-linking in the head region yields dimers that form strong prion strains regardless of the nucleation temperature; conversely, cross-linking in the tail region yields dimers that form weak strains[86]. That the nature of the intermolecular contact determines the nature of the strain explains how these properties can be self-perpetuating, because strains

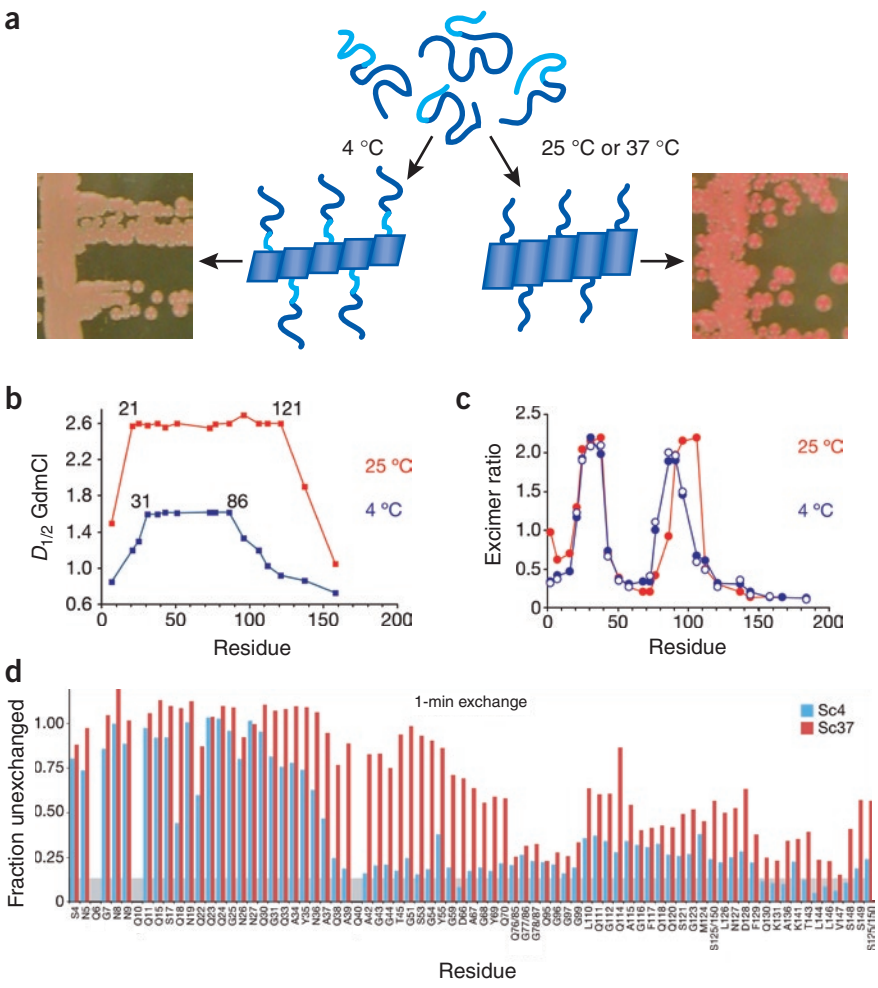

Figure 3 Sup35 nucleates into multiple prion strain variants with unique structural attributes. (**a**) Nucleation of the NM fragment of Sup35 at different temperatures yields unique amyloid conformations that, when introduced into *S. cerevisiae*, induce distinct prion phenotypes visualized by differences in colony color[18,19,86,100]. (**b**) The midpoint of denaturation ($D_{1/2}$) of acrylodan-labeled fibers for each single-cysteine NM mutant reports the size and stability of the amyloid core[86]. (**c**) The excimer ratio, or the ratio of fluorescence at 465 nm relative to that at 375 nm (excitation at 340 nm), of pyrene-labeled fibers for each single-cysteine NM mutant reports the location of residues in self-intermolecular contact (<10 Å)[86]. (**d**) The fraction of unexchanged amide hydrogens (in the presence of deuterium) for most side chains in wild-type NM reports the location and size of the amyloid core. Reproduced from *Nature* (ref. 94).

are propagated from the templating surface. Similar analysis for other prions should determine the generality of these exciting insights into prion strain nucleation.

A recent mutagenesis study has strengthened the idea that prion strain variation is due to differences in the size of the amyloid core[102]. By systematically introducing mutations (proline substitutions or glycine insertions) that destabilize amyloid structures throughout the prion domain of Sup35, King *et al.* found that mutations in largely continuous peptide segments prevented prion propagation *in vivo*, and three prion strain variants showed unique stretches of amino acids (ranging from segments 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 in the Sup35 prion strains studied to date is that they show relatively large structural variations (for example, regions shielded from solvent differ by more than 10 residues). However, Eisenberg and co-workers recently illuminated more subtle structural changes (that do not require substantial changes in solvent exposure) that may also contribute to the unique biological properties of strain variants[85]. Through careful analysis of steric zipper structures of several short peptide fragments from different amyloid-forming proteins, several arrangements of peptides in amyloid-like conformations were identified. Interestingly, the authors found that individual peptide fragments from Sup35 (8-NNQQ-11) and other amyloid-forming proteins can form multiple types of steric zippers (**Fig. 4**). Unfortunately, the large structural differences observed for different Sup35 prion strains[86,94] cannot be mapped to 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. This was recently illuminated through the investigation of Sup35 prions from the yeast species *S. cerevisiae*, *Candida albicans* and *Pichia methanolica*[31]. Each protein efficiently formed self-perpetuating prions when overexpressed, but none cross-catalyzed conversion of 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 (20-mers) that encompass the entire sequence of ScNM and CaNM arrayed on glass slides can be used to interrogate the role of both prion sequence and structural variation on the prion's ability to overcome species barriers. Fluorescently labeled ScNM and CaNM proteins each bound to a small set of their own peptides, but did not cross-react, suggesting that the species barrier is enciphered by small elements of primary sequence. The peptide stretches bound by each protein (ScNM residues 9–39 and CaNM residues 59–86) were named 'recognition elements', and it was observed that each prion protein nucleated into amyloids upon binding to 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].

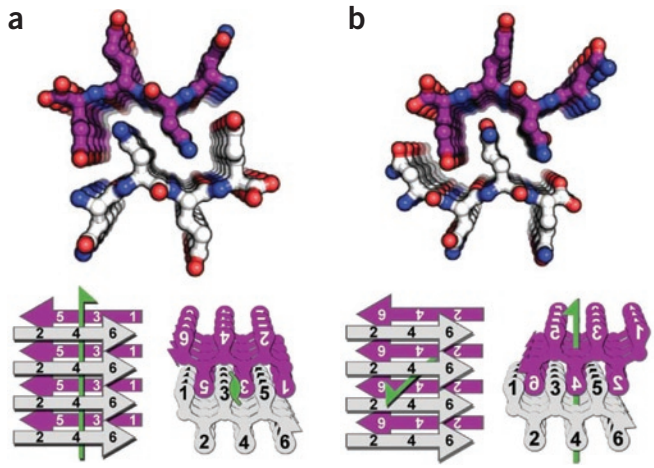

Figure 4 Steric zipper structural variants of a Sup35 peptide fragment. Crystal structures of the Sup35 peptide 8-NNQQ-11 in two of eight possible steric zipper structures[85]. (**a**) The parallel β-sheet steric zipper structure, where the faces of identical peptides in different β-sheets face each other and are both oriented upward. (**b**) A 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 points downward relative to the upward orientation of peptides in the first β-sheet. Reproduced from *Nature* (ref. 85).

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 explored 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 of fiber assembly[29,30] (**Fig. 5**). One conformation of the chimeric prion infects *S. cerevisiae* specifically, whereas the other conformation is specific for *C. albicans*. Using peptide microarrays, the molecular basis of this behavior was elucidated: the monomeric chimera prion bound selectively to peptides in the ScNM sequence at 15 °C, and to CaNM peptides at 37 °C, revealing a remarkable correspondence to the species-specific seeding activities of the two chimeric strains[29]. Thus, selective binding of the chimera to peptides from one species reflects the assembly of chimeric amyloids that selectively infect that same species. These results indicate that nucleation at the recognition elements 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. Spherical, structurally fluid oligomeric structures during NM amyloid assembly have been observed by atomic force microscopy (AFM) and transmission electron microscopy (TEM)[103], and by dynamic light scattering[104]. Several different lines of evidence indicate that these oligomers are on-pathway for amyloid formation[103,105], although conflicting results have been described[106]. Nucleation through the formation of specific intermolecular contacts within molten oligomers provided a completely different explanation for the lag phase in prion assembly than those previously established for the assembly of actin and tubulin[107], and this observation solved the Levinthal paradox[108] for 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 such as NM, it seems 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 timescales.

Other prions and amyloid-forming proteins have since been found to nucleate via similar oligomeric intermediates[13,46,109–114] that are widely posited to be the key toxic species in numerous protein-misfolding diseases[110,111,115,116]. Remarkably, a conformationally specific antibody developed to recognize oligomeric intermediates to the Aβ peptide also recognizes NM oligomers[111,117] and oligomeric intermediates formed by several other proteins[111]. In addition, this antibody inhibits amyloid formation of both NM and full-length Sup35 (ref. 105), confirming that NM oligomers are an obligate intermediate in the nucleation of infectious prion conformers. Nevertheless, 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 NM[118] and polyQ[119], are well suited for such studies.

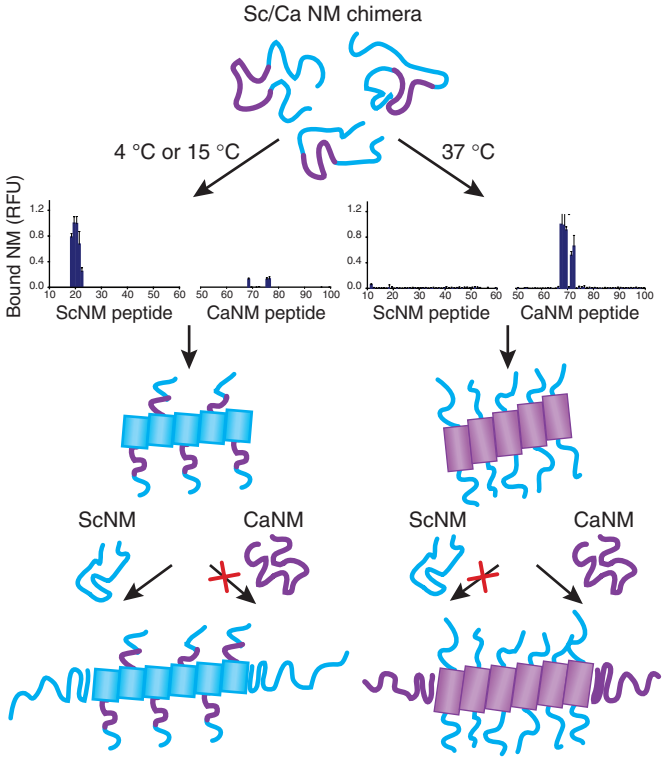

Figure 5 Species-specific infectivities of prion strains. A chimeric Sup35 prion, composed of the N-terminal and middle domains (NM), with sequences from *S. cerevisiae* (Sc, blue) and *C. albicans* (Ca, purple), nucleates into two different prion amyloid conformations at different temperatures with species-specific infectious properties. Peptide microarray analysis revealed that this chimeric prion has two small regions (recognition sequences) that regulate its nucleation behavior, one from the Sc domain and the other from the Ca domain[41]. Low temperatures favor nucleation from the Sc recognition sequence, generating 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.

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 crucial 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[120]. Amyloid formation has also recently been shown to be the basis of melanin production in mammals[121] and the basis of biofilm formation in microorganisms[122], and it seems to have 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 rates[123] 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. Attempts to solve the challenging problems these proteins present in the realm of protein folding should be well worth the effort.

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