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Amyloids and Yeast Prion Biology

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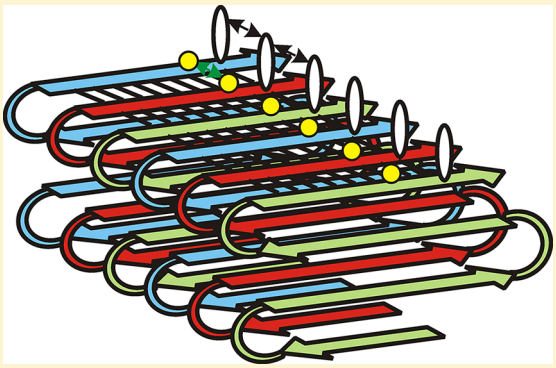
ABSTRACT: The prions (infectious proteins) of *Saccharomyces cerevisiae* are proteins acting as genes, by templating their conformation from one molecule to another in analogy to DNA templating its sequence. Most yeast prions are amyloid forms of normally soluble proteins, and a single protein sequence can have any of several self-propagating forms (called prion strains or variants), analogous to the different possible alleles of a DNA gene. A central issue in prion biology is the structural basis of this conformational templating process. The in-register parallel β sheet structure found for several infectious yeast prion amyloids naturally suggests an explanation for this conformational templating. While most prions are plainly diseases, the [Het-s] prion of *Podospora anserina* may be a functional amyloid, with important structural implications. Yeast prions are important models for human amyloid diseases in general, particularly because new evidence is showing infectious aspects of several human amyloidoses not previously classified as prions. We also review studies of the roles of chaperones, aggregate-collecting proteins, and other cellular components using yeast that have led the way in improving the understanding of similar processes that must be operating in many human amyloidoses.



A "prion" is an infectious protein, able to transmit a trait or a disease without the need for an accompanying nucleic acid. The concept has its origins in studies of the mammalian transmissible spongiform encephalopathies (TSEs), uniformly fatal infectious neurodegenerative diseases.[1,2] TSEs are characterized by the accumulation of an altered form of the cell surface PrP protein, and numerous studies have shown that this protein is the key component of the infectious material. However, recently, evidence has emerged that PrP is not by itself sufficient for full infectivity,[3] and that a phospholipid component is also necessary and affects the specificity of the infectious agent.[4]

The long-known yeast nonchromosomal genetic elements [URE3] and [PSI+][5,6] were shown to be prions of Ure2p and Sup35p based on genetic properties paradoxical for nucleic acid replicons but expected for prions (Figure 1).[7] Specifically, (a) overproduction of the putative prion protein resulted in an ~100-fold increase in the frequency with which the prion arose; (b) even if the prion was cured from a strain, the same prion could (rarely) arise in the cured strain, and (c) the phenotype due to the prion was the same as or similar to the phenotype of a mutant in the gene for the prion protein, and mutants in that gene fail to propagate the prion.[7] The phenotype relation (c) is true if the prion produces effects because of the inactivity of the protein, but not if it produces a novel or toxic effect (as will be seen below for prions [PIN+] and [ISP+] and toxic variants of [PSI+] or [URE3]).

The yeast prions are proteins acting as genes, transmitting information that determines phenotypes both vertically (to progeny cells) and horizontally (by cytoplasmic mixing to a neighboring cell).[7] The mechanism by which a protein transmits genetic information has long been the central puzzle of the prion field, a puzzle to which we have suggested a solution (below and refs 8 and 9). If "epigenetic" is defined as a

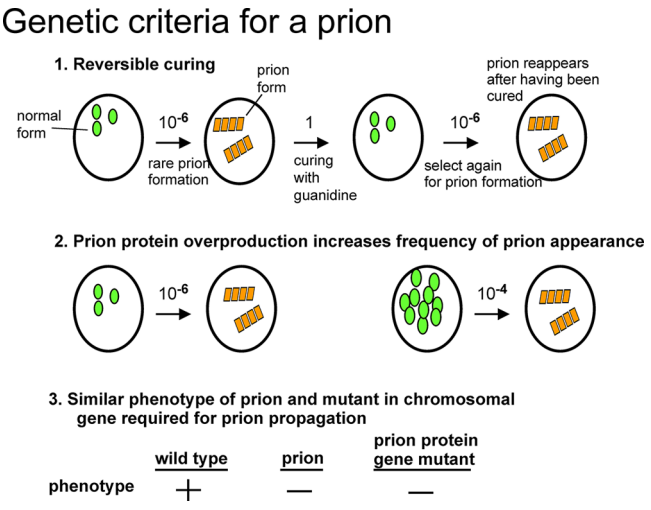


Figure 1. Genetic criteria for a prion.[7] Modified from ref 8.

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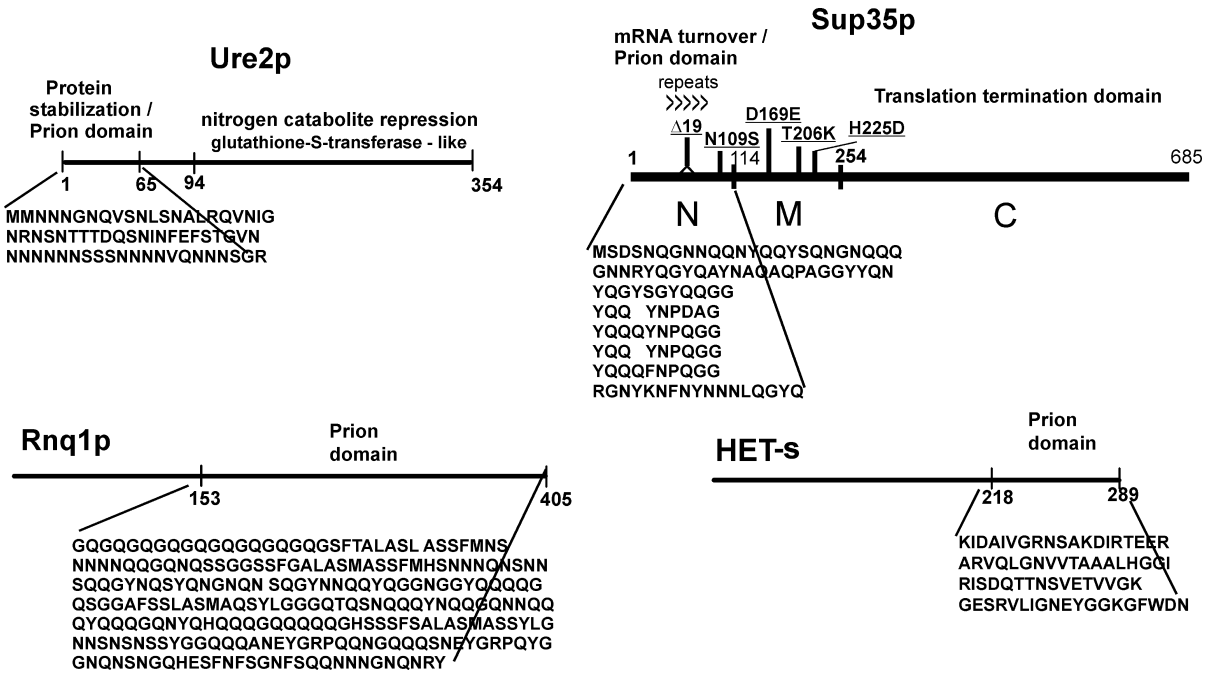

Figure 2. Prion domains[10,11,13] have non-prion functions.[15,172] Prion domains of some other yeast prions have not been as well-defined, and their potential functions have not yet been examined in as much detail as those of Sup35p and Ure2p. The prion domain of Sup35p is roughly residues 1−123 (N), but most studies have used residues 1−254 because including the very ionic M domain (residues 124−254) makes the peptide more soluble and easier to handle biochemically. The locations of polymorphic changes in Sup35p affecting prion transmission are shown.[84] The Ure2p prion domain includes at least residues 1−65, but many studies include the entire unstructured region rich in Q and N, residues 1−89.

heritable change not involving a change in DNA sequence, then yeast prions certainly formally qualify, although in no case have the chromatin changes central to most epigenetic phenomena been implicated in any prion. Here we review the central developments in the yeast prion field, focusing on the structure of prion amyloids, the biology of yeast prions, and, particularly, the relation of the structures to the biology.

PRION DOMAINS

Ure2p is a regulator of nitrogen catabolism, shutting off genes for the enzymes and transporters needed for utilization of poor nitrogen sources when a good nitrogen source is available. Sup35p is a subunit (with Sup45p) of the translation termination factor, stopping translation and releasing the completed peptide from the final tRNA. The parts of Ure2p and Sup35p involved in prion formation and propagation are restricted to their N-terminal domains (Figure 2), both of which are rich in Q and N.[10−12] These regions are called the prion domains, as they are both necessary and sufficient for prion propagation in vivo. The [URE3] prion can propagate in the complete absence of the C-terminal domain,[12] and [PSI+] can propagate with the N-terminal domain of Sup35p expressed separately from the essential C-terminal domain.[10] The Ure2p and Sup35p prion domains are both rich in N and Q residues, as are most yeast prion domains (see below), but the HET-s and Mod5p prion domains are interesting exceptions (see below).[13,14]

The "prion domain" designation may be unfortunate in that it has suggested to some that the sole function of these domains is prion propagation. In fact, the Ure2p prion domain is necessary for protecting the protein from degradation in vivo,[15] and the Sup35p prion domain links the general mRNA turnover systems to translation termination via interactions with the polyA binding protein and the polyA degrading enzyme.[16] While the prion domains are sufficient for prion propagation, deletions of the C-terminal domains of Ure2p or Sup35p dramatically increase the frequency of prion formation,[11,17] perhaps by destabilizing the prion domain.

MOST PRIONS ARE SELF-PROPAGATING AMYLOIDS

Amyloid is a filamentous polymer of protein monomers with a largely β sheet structure in which the β strands run perpendicular to the long axis of the filament (Figure 3). Amyloid is a highly ordered aggregate characterized by relative protease resistance and special dye binding characteristics. Amyloid filaments of many different proteins are prominent features of human diseases, including Alzheimer's disease, type II diabetes, Parkinson's disease, senile amyloidosis, and the prion diseases.

The protease resistance of Ure2p in [URE3] strains,[11] the self-propagating aggregation of Sup35p and Ure2p in the respective prion-carrying strains and in vitro,[18−20] the formation of amyloid by their prion domains,[21−24] and the visualization by electron microscopy of filaments of the prion proteins in prion-containing strains[25,26] all indicated that the prions are amyloid forms of the respective protein. This was conclusively demonstrated when amyloid formed in vitro by recombinant Sup35p or Ure2p or their prion domains were shown to infect yeast spheroplasts with the respective prions.[27−29]

The amyloid filaments of Sup35NM (see Figure 2) grow by addition of monomers to the ends,[30] and either end of a filament can be extended; however, growth from one end tends to be strongly favored.[31,32]

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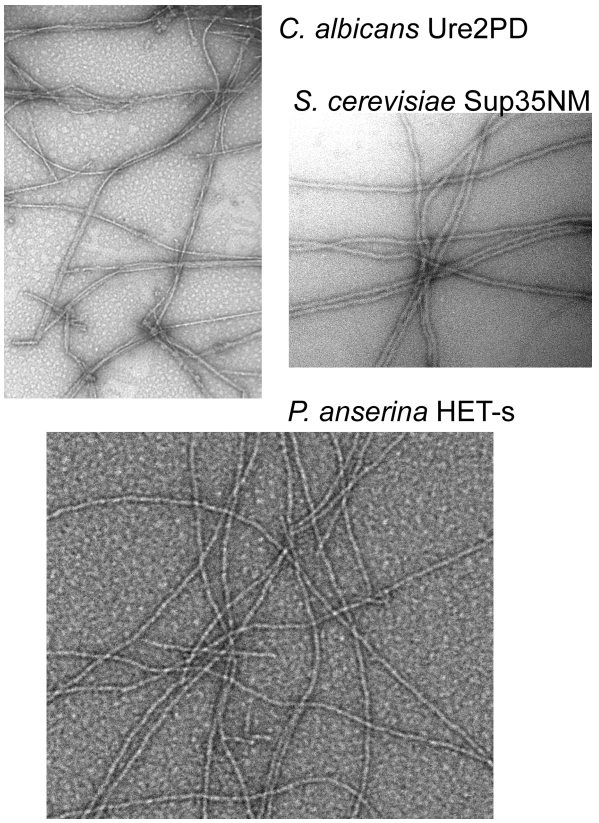

Figure 3. Electron micrographs of negatively stained prion amyloid filaments of the prion domains of Ure2p of *Candida albicans*, Sup35 of *Saccharomyces cerevisiae*, and HET-s of *Podospora anserina*, capable of transmitting [URE3*albicans*], [PSI+], and [Het-s], respectively.

NON-AMYLOID PRIONS

The definition of prion does not require that amyloid be involved, and at least one non-amyloid prion has been found. Zubenko and Jones showed that vacuolar protease B is normally activated by cleavage of its inactive precursor by protease A. However, in the absence of protease A, active protease B can activate its own precursor, although this reaction quickly terminates as cells grow on glucose.[33] However, growing cells on glycerol, which does not repress levels of protease B, allow this self-activation to continue indefinitely.[34] In this system, active protease B acts as a prion, showing nonchromosomal inheritance, arising at increased frequency when the precursor is transiently overexpressed, and requiring the *PRB1* gene for its propagation.[34] This is a prion that is clearly important for survival: in the absence of protease A, cells without the prion die quickly in the stationary phase and are unable to go through meiosis and spore formation.[34]

[GAR] (glucosamine resistant) is a nonchromosomal genetic element making cells resistant to growth inhibition by glucosamine.[35] [GAR] generation is stimulated by overexpression of Std1p and is lost from the double mutant deleted for a part of *PMA1* and for *STD1*.[36] Pma1p is the major plasma membrane proton pump, and Std1p is involved in regulation of glucose metabolism. It is proposed that [GAR] is a functional prion,[36] but neither the mechanism of self-propagation nor the mechanism of glucosamine resistance is understood.

SPECTRUM OF YEAST AND FUNGAL PRIONS

The [Het-s] prion of the filamentous fungus *Podospora anserina* is involved in heterokaryon incompatibility, a recognition of self-phenomenon.[37] Two converging fungal colonies will fuse (form heterokaryons) only if they have identical alleles at approximately a dozen loci (*het* loci). A difference at even one *het* locus results in a programmed cell death and failure to fuse, perhaps to protect against the spread of detrimental fungal viruses.[38] The *het-s* gene has alleles *het-s* (encoding HET-s, the protein whose amyloid is the basis of the [Het-s] prion) and *het-S* (encoding HET-S). Cell fusions between *het-s* [Het-s] and *het-S* cells lead to a programmed cell death. The demonstration of prion infection by amyloid of recombinant HET-s protein (and not by nonspecific aggregates or the soluble protein) was the first such demonstration for any prion.[39] As described below, the similarities and contrasts between the well-studied [Het-s] system and the yeast prion systems have aided in the development of our understanding of the biological and structural aspects of all prions.

The [PIN+] prion was discovered as a nonchromosomal gene needed for [PSI+] inducibility by overexpression of Sup35p[40] and identified as a self-propagating amyloid of Rnq1p, a protein rich in N and Q residues whose deletion produces no phenotype so far identified.[41,42] Overproduction of many proteins with domains rich in Q and N residues can prime [PSI+] formation,[42,43] and several of these proteins, Swi1p and Cyc8p, have proven to form prions themselves.[44,45] Swi1p is a component of a chromatin remodeling complex, and the [SWI+] prion phenotype, poor growth on carbon sources other than glucose, resembles that of *swi1* mutants.[44] Cyc8p, with Tup1p, is a transcription repressor and can form a prion, [OCT+], with a phenotype similar to that of *cyc8* mutations, derepression of a variety of genes.[45]

[ISP+], so named because its phenotype is the reverse of that of [PSI+], antisuppression (decreased read-through of premature termination codons), is a prion of Sfp1p.[46,47] Sfp1p is a transcription factor that promotes transcription of ribosomal protein genes and ribosome biogenesis genes.[48] The [ISP+] prion, unlike *sfp1*Δ, produces an increased level of transcription of the *SUP35* gene, indicating a unique action of the prion and explaining the antisuppressor phenotype.[49] The [ISP+] prion is an amyloid form of Sfp1p; however, the aggregates are located in the nucleus, not the cytoplasm, and the prion is thus only occasionally transmitted by the transfer of cytoplasm from cell to cell (cytoduction).[47]

Recently, using a variant of the Pin screen of Derkatch and Liebman, Tanaka's group has discovered a new prion of *Saccharomyces cerevisiae* that, surprisingly, does not have a prion domain rich in Q and N. [MOD] is an amyloid-based prion of Mod5p, a tRNA isopentenyltransferase.[14] This finding is particularly important because yeast prions are viewed as models of mammalian amyloidoses, only a small fraction of which involve proteins rich in Q and N. The prion core of Mod5p is unusual in its small size and having 6 of 24 residues charged. As with mutation of *MOD5*,[50] carrying the [MOD] prion improves growth in the presence of certain antifungal agents because of the inactivation of Mod5p but slows growth as much in the absence of the drugs as it helps in their presence.[14]

A biochemical screen of yeast proteins rich in Q and N revealed a number of prion candidates, one of which, Mot3p, was shown to form a prion, called [*MOT3*+].[51] Mot3p is a

transcription factor repressing ergosterol biosynthesis and is required for normal vacuolar fusion, and mutants show defective growth at pH 8 and synthetic lethality with a vacuolar protein sorting gene, *VPS41*.[52] A *mot3*Δ strain grows slowly, is respiratory deficient, and is hypersensitive to stress.[53,54] [MOT3+] strains were found to be relatively resistant to Congo Red and Calcofluor White,[51] but other phenotypes have not yet been examined.

PRION VARIANTS: ONE PROTEIN SEQUENCE CAN PROPAGATE SEVERAL DIFFERENT CONFORMATIONS

One prominent feature of mammalian and yeast prions is the existence of prion "strains" or "variants". Scrapie strains with dramatically different incubation periods and distributions of brain lesions were recognized quite early. Variants of the yeast prions with differences in stability of propagation, intensity of phenotype, response to overproduction or depletion of chaperones, or ability to cross transmission barriers due to sequence differences have been described.[55−62] Variants or strains are terms reserved for the same sequence propagating prions with different biological or biochemical properties. Two prions based on different protein sequences are different prions. Variants are clearly due to structural differences between the variant amyloids (e.g., refs 28 and 63), but for no prion variant is the precise amyloid structure known.

STRUCTURAL BIOLOGY BY GENETICS

In an effort to show that the sequence of the Ure2p prion domain was important for its ability to support prion formation, residues 2−89 were randomly shuffled, leaving the amino acid composition and codon usage unchanged.[64] Unexpectedly, each of the five shuffled sequences examined readily formed amyloid in vitro and prions in vivo.[64] The presence of oligopeptide repeats in the Sup35p prion domain had been interpreted as being central to prion formation by that molecule because PrP has similar repeats. It was thus even more surprising that shuffling just the repeats (residues 40−114) or the entire prion domain (residues 3−114) of Sup35p likewise did not prevent prion formation.[65,66] Substituting the Sup35p repeat domain with that of PrP allows prion formation,[67] and shuffling the repeats in such a construct also does not impair prion formation.[66]

This approach has been further used to examine which amino acids favor and which retard prion formation by substituting a segment of one of the shuffled Sup35 prion domains with random peptides and examining the effect on prion formation.[68] Aromatic and hydrophobic residues were favored, and a bias against charged residues was found.[68] No bias for Q or N residues was noted, perhaps because the protein was already so rich in Q and N. Using these results, an algorithm was developed that accurately "post-dicts" the known prion domains rich in Q and N but also was used to design two entirely new prion domains rich in Q and N, which were both shown to, in fact, form prions in vivo.[69] A domain rich in Q and N designed not to form a prion indeed did not.[69]

The shuffling experiments also strongly suggest an in-register parallel β sheet structure for the prion amyloids of Ure2p and Sup35p.[70] The propagation of a prion requires very nearly identical sequence in the prion domain, with just a few changes (or even one in some cases) blocking propagation. Sup35p amyloid filaments (at least) are elongated by the addition of monomers,[30] and this sequence specificity implies that interactions between side chains of incoming monomers must interact with the side chains of molecules already in the chain. In an antiparallel, β helix or out-of-register parallel structure, it is largely nonidentical side chains that are interacting favorably to stabilize the structure. Shuffling the sequence randomly would almost certainly disrupt those interactions; however, if the structure is in-register parallel, then identical side chains are interacting and shuffling disrupts only the order of the interactions, but they can still occur.[70] A row of glutamines or asparagines can form a row of hydrogen bonds linking their side chains, and serine or threonine residues can have similar interactions. Aligned hydrophobic residues can likewise have favorable interactions. In each case, the residues must be aligned for these interactions to occur, so this is the force driving the formation and stabilization of the in-register parallel structure. Of course, charged residues would have unfavorable interactions if in-register, and indeed, the prion domains of Sup35p, Ure2p, and Rnq1p have very few charged residues. This predicted that prion domains, such as those of Ure2p or Sup35p, which can be shuffled and still form prions, are likely to have an in-register parallel architecture.[70]

STRUCTURE OF PRION AMYLOIDS

It has been convincingly argued that almost any protein can be made to form amyloid under some condition (e.g., ref 71), and the structure of amyloid formed from a given protein is very sensitive to the conditions of formation, as well as stochastic factors. Thus, it is imperative that one is studying the structure of the "right" amyloid. Because yeast and fungal prion protein amyloids are highly infectious for yeast cells,[14,27−29,39,72] it is often assumed (by us as well) that the amyloid formed in vitro is primarily the infectious material. The maximal infectivity of amyloid formed from recombinant Ure2p was one-third of that of an extract normalized for the amount of Ure2p,[29] probably not a significant difference in this assay. However, this is far from a demonstration that all particles are infectious. This is thus a qualification of all structural studies to date.

Because of its filamentous nature, amyloid cannot be studied structurally by X-ray crystallography or solution nuclear magnetic resonance (NMR). However, solid-state NMR (reviewed in ref 73) has been particularly useful, along with other methods, in examining amyloids. Solid-state NMR can accurately measure specific interatomic distances by measuring the decay of the signal of specifically labeled proteins, using dipolar recoupling experiments.[74] In an early application of this method to amyloid filaments of a fragment of the Aβ peptide important in Alzheimer's disease, Benziger et al. found that singly labeling the carbonyl carbon of any of several residues of this peptide showed that in each case, the closest ^{13}C neighbor to a labeled atom was ∼5 Å distant, approximately the 4.8 Å expected for an in-register parallel β sheet (see Figure 4).[75] Tycko's group found that full-length Aβ^{1-40} had the same architecture and, using an array of solid-state NMR methods and electron microscopy, determined a detailed structure of these filaments (reviewed in ref 74).

The same methods have been applied to highly infectious filaments of the yeast prion domain peptides, Ure2p$^{1-89}$, Sup35NM, and Rnq1p$^{153-405}$,[76−78] with similar results. For example, the single alanine residue in Ure2p$^{1-89}$ was ∼5 Å from its nearest labeled neighbor, necessarily the same residue on another Ure2p$^{1-89}$ molecule, meaning the molecules were aligned (in-register) at that point. Labeling the carbonyl

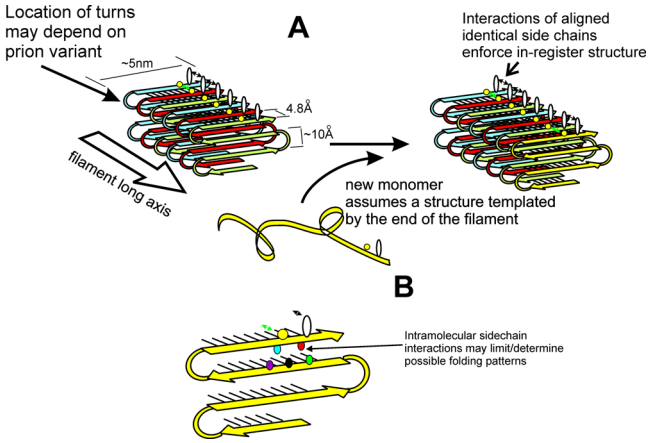

Figure 4. Model of folded in-register parallel β sheet amyloid and how templating of conformation allows prion proteins to act as genes. (A) The favorable intermolecular interactions between identical side chains that keep the structure in-register also force a new molecule, joining the end of a filament, to adopt the same conformation as that of other monomers in the filament. This templating of conformation is analogous to DNA templating its sequence and allows these prion proteins to act as genes. (B) The locations of the folds may be restricted by the nature of the intramolecular interactions between side chains, such as those observed by Eisenberg and co-workers.[185] X-ray fiber diffraction studies of various amyloids show a "cross-β" pattern with a 4.75 Å spacing perpendicular to an ~10 Å spacing,[186] a pattern that has been found to be general for all amyloids. The 4.75 Å spacing is the distance between H-bonded β-strands, while the ~10 Å spacing is the separation distance of stacked β sheets.

carbons of the three leucine residues in Ure2p$^{1-89}$ or the four valine residues gave the same result, indicating that the parallel in-register structure extends through most of the prion domain.[77] Similar results were obtained via labeling of the isoleucine residues in the context of full-length Ure2p: those in the prion domain were in-register.[79] A similar conclusion was reached using electron spin resonance and spin-labels placed every five residues.[80] Labeling the 15 tyrosine residues in Rnq1$^{153-405}$ showed that the nearest neighbor distance was again ~5 Å in each case, and dilution of labeled molecules with unlabeled molecules gave the expected increase in average distance, indicating that the nearest neighbor was in a different molecule.[78] The same type of experiment gave similar results for the carbonyl carbon-labeled tyrosine residues of Sup35NM, all of which are in the N domain.[76] Labeling the eight leucine residues of Sup35NM showed that approximately half were in-register, and at least that fraction was in the β sheet conformation.[76,81] Because only one of the eight leucines is in the N domain, this shows that part of the M domain has the in-register parallel architecture. This result is consistent with evidence that parts of M affect propagation of some [PSI+] variants,[82] that part of the M domain is protected in H−D exchange experiments,[83] that four leucine residues are immobilized in solution NMR experiments,[83] and that residues in M play an important role in the intraspecies transmission barriers to [PSI+] (see below and ref 84).

Mass per length measurements of infectious filaments of Ure2p,[85] Sup35p,[86,87] and Rnq1p[87] give a value of ~1 monomer per 4.8 Å, as expected for an in-register parallel architecture (Figure 4), but not for a β helix, which would produce a value of 0.5 for a two-turn-per-molecule helix (such as HET-s filaments[88]) or lower still for more turns per molecule.

If the prion domains of the yeast prion proteins were one simple wide β sheet, the filaments would be far wider by a factor of ≥4 than they appear on electron micrographs. This implies that the sheet is folded along the long axis of the filament, as shown diagrammatically in Figure 4. The in-register parallel architecture largely specifies the filament structure, but the locations of the folds may vary from one prion variant to another.

Elucidation of the structural differences between prion variants will require seeding recombinant isotope-labeled prion protein with extracts of strains carrying specific prion variants, an approach used by King in studying variants of [PSI+][27] and by Tycko in examining filaments of Aβ seeded by brain tissue from Alzheimer's patients.[89]

Dissenting Views. While the data discussed above establish the in-register parallel architecture of infectious amyloids of Sup35p, Ure2p, and Rnq1p prion domains or, in the case of Ure2p, the full-length protein, there are other views.

Melki's group has consistently argued for a non-β sheet structure of Ure2p filaments with the C-terminal domain as the core structure, and the claim has been made that the protein does not change its secondary structure in forming filaments (e.g., ref 90). However, X-ray fiber diffraction of filaments interpreted to show the absence of β sheet had a dense band of scattering covering the relevant area,[91] and the infectivity of fibrils made by this group has not been reported. Moreover, the secondary structure of the prion domain changes from unstructured[92] to β sheet[24,85,93] upon formation of amyloid. Claims (e.g., ref 94) that Ure2p fibrils are not protease-resistant, do not bind Congo Red (a dye relatively specific for amyloid), and have no increased β sheet content are contradicted by studies by several different groups.[11,24,80,85,95] The notion that the core of the filaments is composed of the C-terminal domain (e.g., ref 96) is contradicted by the ability of amyloid of the N-terminal domain, or this domain fused to various proteins, to efficiently infect yeast.[29] Moreover, the prion domain can propagate [URE3] in vivo in cells not expressing the C-terminal part at all, being efficiently infected by cells expressing the full-length protein and efficiently passing the infection in turn to such cells.[12] Further, as mentioned above, the mass per length measurements of the filaments are indicative of an in-register parallel structure[85] and are incompatible with a model in which a largely unaltered C-terminal domain is the filament core.

Lindquist's group has favored a β helix architecture of Sup35p filaments with head-to-head (N-terminal to N-terminal) and tail-to-tail linkage of adjacent monomers.[97] A large pyrene moiety was attached to a single residue, and a shift in the fluorescence emission frequency was used as an indicator of the proximity of that residue to the same residue in another molecule. However, the pyrene probe is larger than the structure being examined, possibly altering the amyloid structure formed, and the pyrene molecules must be in a specific relative orientation, possibly precluded by the structure of the amyloid, giving deceptive results. Moreover, deletion of the entire "tail" region does not interfere with the propagation of many [PSI+] variants (e.g., ref 27) and mass per length measurements indicate one monomer per 4.7 Å,[86,87] while the β helix model demands a value half or less that observed, as stated above. Further discussion of these apparent disagreements may be found elsewhere.[98]

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While the physical evidence is sufficient to show that the Ure2p, Sup35p, and Rnq1p amyloids are in-register parallel structures, this architecture also provides an explanation of how prion variant information can be propagated. An explanation for this central phenomenon has not been put forward on the basis of either the β helix or Ure2C-as-core models.

MECHANISM OF CONFORMATIONAL TEMPLATING

A single prion protein with a single amino acid sequence can be the basis for a wide array of prion strains or variants. This is perhaps the central mystery of prions and is one reason many did not believe that a protein could be the carrier of an infection. The folded parallel in-register architecture of the yeast prion amyloids suggests a mechanism for explaining the ability of prion filaments to template their own conformation (Figure 4).[8]

The positive interactions between identical amino acid side chains (hydrogen bonds between aligned N, Q, S, or T residues; hydrophobic interactions between aligned I, V, L, F, W, or Y residues) keep the structure in register, because these interactions can happen only if the identical side chains are aligned. Charged residues would have unfavorable interactions but are rare in the prion domains with this architecture. These same interactions force the unstructured prion domain of the monomer joining the end of the filament to assume the same conformation as that of monomers already in the filament (Figure 4). The turns of the strand (at the location of the folds of the sheet) of the molecule joining the filament must be in the same location as for the molecules already in the filament. If turns are in different locations in different prion variant amyloids, this mechanism can explain how each conformation (the basis of each variant) is propagated. These same interactions can reproduce, in molecules newly joining the end of the filaments, the extent of the β sheet region and the assembly of protofilaments into multimers. The latter is the basis of the difference between two structural variants of Aβ protein amyloid.[73]

ROLES OF CHAPERONES AND OTHER CELL COMPONENTS IN PRION GENERATION, PROPAGATION, AND SEGREGATION

Although highly infectious amyloids of the yeast prions form from purified recombinant proteins spontaneously in vitro, there are many cellular components that affect their generation, propagation, and segregation in vivo.

Hsp104 Is Needed for Propagation by All Amyloid-Based Prions. Hsp104 is a disaggregase that works with Hsp70s and Hsp40s on denatured proteins[99] and is specifically inhibited by millimolar concentrations of guanidine in the growth medium.[100−102] Hsp104 is required for the propagation of [PSI+][103] and all other amyloid-based yeast prions.[14,40,44−46,51,104] When Hsp104 is inhibited, prion filaments continue to grow longer but are not split to make new filaments, so that as cells divide, the number of filaments per cell decreases until some cells with no filaments are produced, meaning that they are cured of the prion.[18,105−107] Thus, Hsp104 is needed for prion propagation for the regeneration of seeds by breaking long amyloid filaments into short ones (reviewed in ref 108). The filament breakage reaction in vitro has been shown by several groups to require Hsp70s and Hsp40s in addition to Hsp104,[109−111] as was previously found for the in vitro disaggregation reaction.[99] In vivo experiments support the involvement of Hsp70s and Hsp40s in filament breakage.[112] The Hsp104 homologue from *Escherichia coli*, ClpB, can substitute for Hsp104 in prion propagation only in the presence of the *E. coli* Hsp70 homologue, indicating that the two proteins interact in the filament breakage reaction.[112] Hsp104 disaggregates proteins by pulling monomers out of the aggregate through a pore in the Hsp104 hexamer.[113,114] The Hsp104 machinery apparently acts the same on prion filaments, extracting a prion protein monomer from the middle of the filament, thereby breaking it into two filaments[114] (Figure 5).

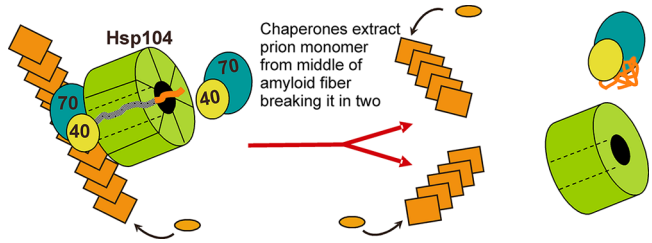

Figure 5. Chaperones Hsp104, Hsp70, and Hsp40 cooperating to extract a monomer from the middle of a prion filament thereby break yeast prion amyloid filaments to make new seeds (modified from ref 187).

Overproduction of Hsp104 Cures Only [PSI+]. Upon overproduction of Hsp104, the [PSI+] prion is cured,[103] albeit not as rapidly as when the activity is inhibited with guanidine (reviewed in ref 108). The mechanism of curing is apparently not the simple solubilization of the amyloid filaments, as filaments formed before Hsp104 is overproduced remain and actually grow in size.[107] Certain N-terminal mutations of Hsp104 eliminate curing of [PSI+] by overexpression of Hsp104 but do not affect prion propagation or sensitivity to heat shock.[115] The Hsp40 group chaperone Sis1p is necessary for [PSI+] propagation,[116] but some Sis1p functions are dispensable for [PSI+] propagation but required for curing of [PSI+] by overexpression of Hsp104.[117] The curing is dependent on the ubiquitin system, but not prion propagation (and Sup35p is not detectably ubiquitinated).[118] Hsp104 overproduction curing of [PSI+] requires the function of Sti1p and Cpr7p (two cochaperones that coordinate Hsp90s with Hsp70s), but neither Sti1p nor Cpr7p is needed for prion propagation.[119,120] Thus, while the mechanism of Hsp104 overproduction curing [PSI+] remains unclear, it is unlikely to be simple resolubilization and/or renaturation of Sup35p from filaments and is clearly not due to Hsp104 acting alone.[108]

Recently, a binding site for Hsp104 in Sup35M (residues 129−148) has been identified.[111] Deletion of this short region does not prevent propagation of [PSI+] or its dependence on Hsp104 but does make [PSI+] incurable by overproduction of Hsp104.[111] It should be noted that overproduction of Hsp104 also stimulates the generation of [URE3], particularly by *Candida albicans* Ure2p.[121]

Hsp104, Damaged Protein Segregation, and Prion Segregation. On mitotic division of yeast, most oxidatively damaged or aggregated proteins are retained in the mother cell.[122] This system requires Sir2p,[122] a histone deacetylase essential for normal replicative longevity in yeast.[123] Hsp104 is found to be associated with aggregated oxidized proteins, and overexpression of Hsp104 can largely compensate for the

defective retention of such aggregates in the mother cell and the decreased lifespan of sir2Δ mutants.[124] These results might suggest that this system is involved in the curing of [PSI+] by overproduction of Hsp104, but aged cells do not have elevated frequencies of [PSI+] or [URE3].[125] Nonetheless, this system might be involved in handling prions.

Hsp70s and Hsp40s Have Diverse Roles in Prion Propagation. The Hsp70 family in yeast includes soluble Ssa1p, Ssa2p, Ssa3p, and Ssa4p and ribosome-bound Ssb1p and Ssb2p.[126] Overexpression of Ssa1p (a cytoplasmic soluble Hsp70) inhibits curing of [PSI+] by overexpression of Hsp104;[127] a mutant of Ssa1p results in the failure to stably propagate [PSI+],[128] and mutation of Ssa2p can result in the loss of [URE3].[129,130] Although Ssa1p and Ssa2p are nearly identical, they affect [PSI+] and [URE3] differently, a specificity that is determined by a single methyl group (Ala vs Gly at residue 83).[131] Hsp70s are involved in Hsp104's filament breakage reaction, as discussed above, and also appear to be involved in the curing of [PSI+] by Hsp104 overproduction.[108]

While the Ssa's generally promote prion propagation, the Ssb's have opposite effects. The level of prion generation is increased in *ssb1*Δ *ssb2*Δ strains, and Hsp104 overproduction curing of [PSI+] is enhanced by also overproducing an Ssb protein.[132]

The Hsp40s Ydj1p and Sis1p affect prion propagation, and an array of evidence indicates that these cochaperones act by influencing Hsp70 activities (reviewed by Reidy and Masison[108]).

Btn2 and Cur1 Promote Aggregate Collection and Prion Curing. Cells have developed an array of mechanisms to deal with errant proteins, those that are misfolded, inappropriately aggregated, or both. The ubiquitin−proteasome system and vacuoles degrade many proteins, and aggregates are sequestered at special cellular sites. *BTN2* and *CUR1* were isolated in a screen for high-copy number plasmids producing curing of [URE3].[59] Cells from which both genes had been deleted had a higher [URE3] prion seed number than the parent and showed partial resistance to prion-curing agents and an increased proportion of "strong" [URE3] variants on de novo prion induction, suggesting that the normal levels of these proteins are also acting to limit prion propagation. In the process of curing [URE3], Btn2p and Ure2p prion aggregates show striking colocalization at a single site in the cell, analogous, it was suggested, to the mammalian aggresome.[59] Neither Btn2 nor Cur1 overexpression cured [PSI+], perhaps because the seed number of [PSI+] is higher than that of [URE3].

Btn1p was first identified as a homologue of human Cln3p, mutant forms of which cause Batten's disease, involving neuronal accumulation of certain complex lipids.[133] The level of *BTN2* transcription was reported to be elevated in *btn1*Δ cells, but this has not been a consistent finding. A dramatic elevation of the level of *BTN2* and *CUR1* expression occurs on heat shock or several other stress conditions.[134] Neither protein is a member of the recognized heat shock protein families, but both are somewhat homologous to each other and to the HOOK proteins,[59,135] involved in endocytosis in *Drosophila* and mammalian cells. HOOK proteins in both flies and mammals bind to HOPS vacuolar sorting complex proteins (see the references in ref 136). Btn2p interacts with the yeast endocytic SNARE (membrane fusion) complex and the retromer complex (that coats vesicles from late endosomes headed for the Golgi).[136] A *btn2*Δ mutant mislocalizes Yif1p (a membrane protein required for fusion of endoplasmic reticulum-derived vesicles with the Golgi) to the vacuole instead of the Golgi.[136,137]

Another study confirmed the prion curing activity of overproduced Btn2p and Cur1p and showed that both bind to Sis1p, an Hsp40, bringing it into the nucleus.[134] The depletion from the cytoplasm of Sis1p was suggested as the cause of prion curing,[134] as Sis1p is needed for propagation of all yeast prions studied.[116]

There are two cellular sites at which aggregates accumulate (prion or otherwise): one juxtanuclear and the other peripheral.[134,138,139] Hsp42 is necessary for gathering of aggregates at the peripheral site;[139] Btn2p binds to Hsp42, and that binding is necessary for Btn2p's concentration in the peripheral site.[134] Studies of these aggregate accumulation sites have often assumed that overproduced aggregated prion-forming proteins, such as Ure2p, or the prion domains of Sup35p or Rnq1p are actually prions and have concluded that they are observing collection of prion particles at these sites.[134,138] However, while overproduced prion proteins generally aggregate in most cells, only a tiny minority of cells go on to develop the corresponding prion (e.g., ref 7), so those studies do show that aggregates of prion proteins are moved to the aggregation sites, but not necessarily the prion particles themselves. In contrast, Kryndushkin et al. used cells in which Ure2p was not substantially overproduced and which were known to carry the [URE3] prion; therefore, the aggregates observed were prion aggregates, and their colocalization with Btn2 at a single cellular site shows localization of prion particles.[59]

In addition to model unfolded proteins and prions, Btn2p has an effect on certain non-amyloid aggregates. Optineurin mutations are associated with amyotrophic lateral sclerosis, and expression of optineurin, particularly those mutants, is toxic for *S. cerevisiae*.[140] Overproduction of Btn2p relieves and *btn2*Δ exacerbates this toxicity, and optineurin aggregates coincide perfectly with Btn2p, indicating a role for Btn2p in relieving the toxicity of these aggregates.[140]

It is clear that Btn2p and Cur1p have a physiological role in responding to abnormal protein aggregates, segregating them from the rest of the cell, much as does the mammalian aggresome. The mammalian HOOK proteins, homologous to Btn2p and Cur1p, are not yet known to have such a function. Btn2p has another function, in endocytosis and the movement of vesicles between organelles. It will be of interest to know if these functions are two aspects of a single activity.

■ ARE YEAST PRIONS A BANE OR A BENEFIT?

Because mammalian prions are uniformly fatal, there has never been a doubt that they are diseases. If yeast prions were uniformly fatal, they would probably not have been discovered. We will see below that there are indeed lethal forms of yeast prions. The fact that the [Het-s] prion is necessary for an apparently normal fungal function, heterokaryon incompatibility,[38] led us to suggest that this was the first example of a beneficial prion.[141] It was then reported that the [PSI+] prion made yeast cells more resistant to heat or high ethanol stress, suggesting a benefit to the host,[142] but subsequent studies did not support this conclusion.[143] Rather, it was found that of the dozens of conditions tested, there were none that always favored [PSI+] over [psi−] cells.[143] When differences were noted, three-fourths of such differences were [psi−] growing

better than [PSI+], but the genetic background made these results variable. However, even the minority of cases in which a particular genetic background−growth condition combination favored [PSI+] over [psi−] could not be reproduced by another group using the same strains.[144] It is reported that certain stress conditions induce the appearance of [PSI+] in a strain with a Sup35 prion domain modified to make it more likely to convert to the prion form.[145] However, those conditions did not induce [PSI+] generation in a strain with the normal Sup35 prion domain.[146]

Without a dramatic benefit, however, it may be impossible to answer the question of "bane or benefit" by this approach. Even if a modest benefit or induction of [PSI+] under some condition were reproducible, it would be nearly impossible to ascertain what is the representation of such condition in the ecological niche of yeast. Other approaches are needed.

[PSI+] and [URE3] Are Rare in Wild Cells, but [Het-s] Is Common. Even a detrimental or lethal virus, plasmid, or prion can be found in the wild, because the spread of the infectious element outruns the damage done to the host. Chronic wasting disease is a uniformly fatal prion disease found in ∼10% of wild elk and deer in parts of the western United States.[147] Certainly, a beneficial prion (or plasmid or virus) would be found in most wild individuals, and a prion being rare would be clear evidence that it is detrimental. We surveyed 70 wild *Saccharomyces* isolates and found that while all of the several parasitic nucleic acid replicons were found in some fraction of the strains, none had [URE3] or [PSI+], and only 11 of 70 had [PIN+].[148] A larger more recent survey confirmed the rarity of [PSI+] and gave an even lower estimate of the incidence of [PIN+], although [URE3] was not tested.[149]

All of these infectious elements of *S. cerevisiae* spread only by outcross mating, so the validity of the argument in the previous paragraph relies on the degree to which such matings occur. Previous estimates of this frequency, using population genetic methods, were that such matings occur only once or twice per 10^5 mitotic doublings or, as one author states, only approximately once per 100 years.[150,151] We re-examined this issue from a different viewpoint. An infectious element found in wild strains spreads only by outcross mating, and this spread must balance the harm caused to the host to give the observed incidence[146] (Figure 6). The 2μ DNA plasmid is known to slow cell growth by 1−3%,[152−154] an effect that we confirmed.[146] Nonetheless, 2μ DNA was found in 38 of 70 wild isolates,[148] indicating that yeast must mate at least once per 100 mitotic doublings or 1000 times more often than had previously been estimated.[146] The same equations imply that a prion found in 1% of wild strains must impart a >1% growth/survival defect on its host.[146] This constitutes a substantial detriment in the wild, but it must be noted that this is the detriment of the mildest form of each prion. As discussed below, lethal and near-lethal variants of [PSI+] and [URE3] comprise a substantial proportion of the respective prion isolates, and of course, these confer a much greater detriment on the host.

The [Het-s] prion of *P. anserina* is found in >90% of wild isolates with the *het-s* allele,[155] as one would predict for this apparently adaptive prion. However, the [Het-s] prion also is involved in a meiotic drive system, in which sexual crosses of the type female *het-s* [Het-s] × male *het-S* result in the lethality of spores carrying the *het-S* allele.[156] Whether the [Het-s] prion is a net benefit through its heterokaryon incompatibility function or a net detriment through meiotic drive, one can say that the HET-s protein is certainly evolved to be a prion. A protein evolved to be a prion should have only one prion variant, adapted to its function, and that is true of [Het-s] (reviewed in ref 38). This contrasts with the yeast prions, each known to have multiple variants with dramatically different properties. A knee only bends in one way, but there are many ways to break a leg.

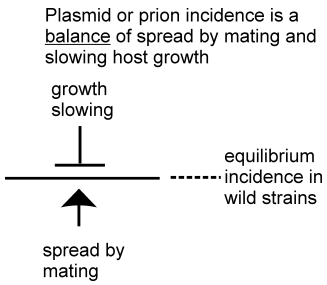

Figure 6. Incidence of a nonchromosomal genetic element (infectious element) in yeast determined by a balance between spread by outcross matings and the slowing (or speeding) of growth (or survival) of the host resulting from the infection. The scarcity of even the mildest yeast prion variants in the wild implies they are detrimental to their hosts.[146,148] The 2μ DNA plasmid is known to slow mitotic growth 1−3%,[152,154] spread only by outcross mating, and is rarely lost. Thus, *S. cerevisiae* must have an outcross mating ∼1% of mitotic divisions.[146] With the knowledge of outcross mating frequency, the mildest form of [PSI+] must slow cell growth >1% because it is less abundant than 2μ DNA in wild strains.[146]

Lethal Variants of Yeast Prions. The variants of [PSI+], [URE3], or other prions studied by yeast geneticists have been, necessarily, those that are not lethal to their hosts. For example, the translation termination activity of Sup35p is essential for cell growth, and an amyloid of Sup35p whose affinity for the soluble form of the protein was sufficiently high would inactivate too much of the protein and kill the cells.

To isolate such "suiψdal [PSI+]" we constructed a strain in which a *URA3 CEN* plasmid expressed a low level of Sup35C, lacking the prion domain, but able to support growth of a cell.[157] Cells with suiψdal [PSI+] would now survive but could not afford to lose the plasmid expressing Sup35C. This revealed that more than half of [PSI+] isolates in this screen were either suiψdal [PSI+] or extremely sick, forming only tiny colonies.[157] However, such a screen recovers only a fraction of lethal prions, those that depleted Sup35p too much. A prion whose amyloid had a toxic action on the cell, disrupting some important process, would kill the cells even if Sup35C were supplied.

Ure2p is not an essential protein, and in some genetic backgrounds, a *ure2*Δ does not even slow cell growth. Using such a strain, we noted that many very slow-growing [URE3] isolates could be found on selective media. These strains recover normal growth when they lose the [URE3] prion. Thus, there are clearly toxic prions, and future work will be directed to understand the mechanisms of such toxicity.

It should be noted that several other lines of evidence point toward toxicity of a [PSI+] variant that would normally be relatively mild. [PSI+] decreases the fitness of *rkr1*Δ cells defective in protein quality control.[158] Certain mutations in the Hsp40 Sis1p do not impair its essential function but do result in [PSI+] being lethal for the cell.[117] The combined presence of [PSI+] and [PIN+] is lethal in *sla2*Δ cells.[159] Overexpression of Sup35NM is lethal in [PSI+] cells because, like suiψdal [PSI+], it sequesters the Sup35p, while overexpression of full-length Sup35p is lethal in [PSI+] cells because of sequestration of all of the Sup45p, the other subunit of the translation termination

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factor.[160] [PIN+] toxicity likewise becomes evident upon overexpression of Rnq1p, in this case by sequestration of Spc42, a component of the spindle pole body.[161] These phenomena are suggestive of possible mechanisms that may be operative in the toxicity of yeast prions without other mutations or protein overexpression conditions.

MOD5 encodes a tRNA isopentenyltransferase, and *mod5*Δ mutations slow cell growth but make them resistant to antifungal drugs of the fluconazole group that block ergosterol biosynthesis.[162] The *mod5*Δ mutants use fewer isopentenyl donor units, freeing it for use in ergosterol biosynthesis.[162] The [MOD] prion, by inactivating the prion protein, Mod5p, has the same effect.[14] It seems unlikely that fluconazole drugs are sufficiently abundant in the yeast ecological niche to make this resistance sufficiently advantageous to offset the growth defect. The growth of cells in the presence of fluconazole enriches [MOD]-carrying cells, but it is not clear whether it induces the appearance of the prion as suggested.[14]

Prion Forming Ability Is Not Conserved. The prion domains of Sup35 proteins of several non-*S. cerevisiae* yeasts can support [PSI+] formation in *cerevisiae* when fused to the *cerevisiae* Sup35C,[163−166] suggesting that prion forming ability is conserved, often interpreted as evidence that prion forming ability is an advantage. However, [URE3] formation occurs in some non-*cerevisiae* species, but not in others.[61,167−170] For example, the Ure2p of *C. albicans* can form a [URE3] prion in *S. cerevisiae*, but that of *Candida glabrata* cannot, even though the latter is more closely related in sequence to *cerevisiae* Ure2p[170] and Ure2p of *Saccharomyces castellii* could not form [URE3] or be infected by that of other *Saccharomyces* species.[61] Using *S. cerevisiae* as a test bed for prion formation is certainly much easier than engineering other species to reveal prion formation, but the variation in the properties of the chaperones and other cellular components involved in prion generation and propagation makes this a risky business. Notably, Aigle's group showed that the Ure2p of *Kluyveromyces lactis* could not form a prion at detectable frequency in *K. lactis* itself,[169] and Ure2p of *C. glabrata* cannot form [URE3] in *C. glabrata*.[171]

Prion Domains Have Non-Prion Functions. The N-terminal prion domain of Sup35p is not necessary for the essential translation termination function[10] but is necessary for the normal mRNA turnover process, regulating the shortening of the 3′ polyA structure of mRNAs by interacting with the polyA binding protein and polyA-degrading nucleases.[16,172] The Ure2p prion domain likewise has a function in stabilizing the protein from degradation.[15] Thus, conservation of the sequence of prion domains is not an argument for selection for prion forming ability. It could simply be selection for the normal (non-prion) function of the prion domain. Furthermore, as discussed above, randomly shuffling the prion domain of Sup35p or Ure2p does not prevent prion formation.[64,65] The sequence of these prions is not important for prion formation, just the amino acid content, so that even if there were selection for prion forming ability, it need not result in conservation of sequence.

Prion Infection Induces Hsp's. One indication of whether yeast prions are beneficial or detrimental is the cellular reaction to infection. Introduction of [PSI+] or [URE3] induces heat shock proteins Hsp70 and Hsp104, suggesting that the cell views the prions as a stress.[128,130] Sgt2p modulates effects of chaperones on yeast prions and is proposed to be a "sensor" of amyloid.[173] Sgt2p, a protein that helps in insertion of proteins into the ER membrane, is induced upon infection with [PSI+] or [PIN+] and interacts with prion proteins, possibly directing prion aggregates to chaperones.[173] These reactions of the cell to prion infection suggest that the cell considers these prions a problem, not a benefit.

Intraspecies Barriers to [PSI+] Transmission. Wild strains of *S. cerevisiae* have an array of Sup35p prion domain sequences, roughly divided into the reference sequence (found in lab strains), the Δ19 sequence with a 19-amino acid deletion in the prion domain, and E9 with several changes in the M domain and the N109S change.[84] Each of these polymorphs is capable of becoming [PSI+], but each presents a barrier to transmission of [PSI+] from either of the other polymorphs.[84] The rare wild [PSI+] isolates are sensitive to these barriers, indicating that they are effective in blocking the propagation of [PSI+].[62] The heterozygosity for the residue 129 M/V polymorphism of human PrP prevents infection with Kuru or other forms of human TSE, and Mead et al. have suggested that this protection from prion infection has produced this polymorphism.[174] We proposed a similar explanation for the polymorphism of the Sup35p prion domain.[84]

Yeast prion variants are most commonly classified as "weak" or "strong", although, as mentioned above, they may differ in many different properties. How well these other properties correlate with the weak versus strong classification has not been widely studied. Via examination of the intraspecies barriers to transmission of a strong [PSI+] variant, it has been found that any of four patterns of transmission to the common polymorphs of Sup35p may occur.[62] Interestingly, simple extended mitotic growth of one such transmission variant results in generation and segregation of other transmission variants, without changing the strong character of the [PSI+].[62] The rare wild [PSI+] isolates[149] were all weak but displayed the same array of transmission variants as the single strong strain studied.[62] These results were interpreted as support for the "prion cloud" model proposed for mammalian prions by Collinge and Clarke[175] and Weissmann and co-workers,[176] in which a continuing process of generation of new variants and their segregation during cell growth is envisioned. The results also have important implications for structural studies: homogeneity on the strong versus weak scale of cells transfected with a preparation of amyloid does not imply homogeneity of structure even of the fraction of the filaments that are infectious.

■ AMYLOIDOSES, STRUCTURE, AND YEAST PRIONS

Like the yeast prions examined to date, most amyloids involved in human diseases are found to be in-register parallel β sheet structures (reviewed in ref 98). The only known exception so far is the amyloid of transthyretin whose structure is not known but is clearly not in-register parallel.[177] Transthyretin is a serum protein that transports thyroid hormone and retinol binding protein. Amyloid of transthyretin is the basis of senile amyloidosis and several inherited amyloidoses. In contrast, none of the known functional amyloids are known to have this architecture (e.g., refs 178−180). Pathological amyloids, including most prions, are known to be highly polymorphic, but functional amyloids or prions should preferentially adopt a single structure. Pmel17, a melanocyte protein that functions as an amyloid in melanin biogenesis, appears to be an exception to this rule based on its polymorphism when amyloid formation occurs in vitro,[180] but whether it is polymorphic in vivo remains to be determined.

PROSPECTS

There are undoubtedly many more prions to be found, particularly in organisms other than the intensively studied *S. cerevisiae*. Increasingly, infectious aspects of human amyloid diseases are being uncovered,[181,182] and many laboratories now use yeast as a testing bed for finding possible mechanisms of non-prion amyloid toxicity (e.g., ref 183). The use of yeast to screen for anti-prion therapeutic agents has produced some promising candidates, and suggestions that a chaperone activity of ribosomes may play a role in prion propagation.[184] Among many other outstanding issues are (i) the exact structural differences among different prion variants, (ii) mechanisms of toxic prion variants, (iii) mechanisms of Btn2 and Cur1 prion curing and the sites to which they move prion particles, (iv) mechanisms of Hsp104 overproduction curing of [PSI+] and why it is specific for [PSI+], and (v) while the well-studied [PSI+], [URE3], and [PIN+] appear to be diseases, whether there are functional yeast prions, like [Het-s] of *Podospora*.

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