Creutzfeldt-Jakob disease and mad cows: lessons learnt from yeast cells

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Summary

Transmissible spongiform encephalopathies are fatal neurodegenerative diseases that affect mammals including humans. The proteinaceous nature of the infectious agent, the prion, and its propagation, challenge established dogmas in biology. It is now widely accepted that prion diseases are caused by unconventional agents principally composed of a misfolded host-encoded protein, PrP. Surprisingly, major break-throughs in prion research came from studies on functionally unrelated proteins in yeast and filamentous fungi. Aggregates composed of these proteins act as epigenetic elements of inheritance that can propagate their alternative states by a conformational switch into an ordered β-sheet rich polymer just like mammalian prions. Since their discovery prions of lower eukaryotes have provided invaluable insights into all aspects of prion biogenesis. Importantly, yeast prions provide proof-of-principle that distinct protein conformers can be infectious and can serve as genetic elements that have the capacity to en- cipher strain specific information. As a powerful and tractable model system, yeast prions will continue to increase our understanding of prion-host cell interaction and potential mechanisms of protein-based epigenetic inheritance.

Key words: BSE; Creutzfeldt-Jakob; neurodegeneration; prion; transmissible spongiform encephalopathy

Prion diseases: rare and fatal diseases of mammals

Prion diseases or transmissible spongiform encephalopathies (TSEs) are infectious neurodegenerative diseases that are characterised by proteinaceous deposits and spongiform changes in the central nervous system (CNS) [1]. Astro- and microgliosis, the lack of a lymphocytic inflammatory response and the accumulation of an aggregated host-encoded protein termed prion protein are histopathological features of TSEs. Prion diseases were first identified in the 18th century as a trembling disease in sheep and goats in France and Germany [2] (table 1). Since then, prion diseases have been reported in deer and elk (chronic wasting disease), cattle (bovine spongiform encephalopathy) and other mammals [3]. Human prion diseases can be aetiologically classified into three subsets: sporadic, heritable and acquired or iatrogenic. Sporadic prion disease in humans is known as Creutzfeldt-Jakob disease (CJD) and accounts for 85% of all human TSE cases with an incidence of approximately 1–2 per million of the population [4]. Heritable prion diseases such as fatal familial insomn- ia and Gerstmann-Sträussler-Scheinker-Syndrome, can be attributed to approximately 30 pathogenic mutations in the prion protein gene [5]. Iatrogenic transmission of CJD has occurred in humans due to the use of TSE-infected surgical instruments, organ transplantation from TSE-donors or through the administration of growth hormones from a TSE-donor [6]. Cases of naturally acquired prion disease include Kuru, a disease of the South Fore tribe of New Guinea, and a new variant of CJD (vCJD) [7, 8]. The outbreak of mad cow disease or bovine spongiform encephalopathy (BSE) in the UK in the mid-1980’s resulted in more than 180,000 cases of BSE with an estimated 1 million cattle infected during the BSE epidemic [9]. In 1989, meat and bone meal was postulated as the source of the infection and was subsequently banned as a food supply for ruminants. In 1994 the first case of human vCJD was reported and it is likely to have been caused by dietary exposure to BSE contaminated beef [10]. To date only 222 cases of vCJD have been reported but since 2004, four cases of vCJD were detected and linked to blood transfusions from pre-clinical TSE-donors raising concerns on the possibility of infection by silent carriers of the TSE agent [11] (table 1).
Protein aggregates as infectious entities

Studies by Griffith in the 1960’s showed that the TSE agent was resistant to all known methods of inactivating nucleic acids, which led to the suggestion that the infectious TSE agent is devoid of a pathogen coding nucleic acid [12]. In 1982 Bolton and colleagues isolated an aggregated and misfolded form of a cellular protein that co-purified with infectivity from scrapie infected hamster brains [13]. The native cellular isoform of the protein was termed PrP\(^{C}\) or the prion protein and the isoform associated with infectivity was termed PrP\(^{Sc}\). Prusiner postulated that the infectious agent is composed predominantly of the misfolded isoform of the prion protein and is capable of self-propagating by converting endogenous PrP\(^{C}\) to PrP\(^{Sc}\) [14]. It is now widely accepted that prions represent protein-based infectious entities that replicate by a process called seeded polymerisation. According to this model, a seed of polymeric fibrillar PrP\(^{Sc}\) serves as a template for the conformational conversion of PrP\(^{C}\) to its pathogenic isoform (fig. 1A). The mechanism of seeded polymerisation is shared by other disease-associated proteins, including A-beta (Alzheimers disease) and huntingtin (Huntington’s Chorea) that form highly ordered \(\beta\)-sheet rich fibrils termed amyloid [15]. Importantly, self-seeding of ordered protein aggregates is necessary but not sufficient for prion propagation. To increase the number of infectious seeds fibril growth must be followed by fibril fragmentation. Recent data has shown that the most infectious prion particle consists of approximately 14–28 monomers of PrP [16]. The mechanism of prion shearing or fragmentation in mammals is still unknown and no cellular factors have yet been identified to be categorically involved in this process.

The prion protein

The host-encoded PrP\(^{C}\) is a 253 amino acid (human; other species vary) protein with two sites for glycosylation, a glycosylphosphatidyl-inositol (GPI) anchor and is trans-located to the plasma membrane [17]. PrP\(^{C}\) is present in most mammalian cells but is enriched in the cells of the CNS and of the immune system [18]. The exact function of PrP\(^{C}\) is unknown, however PrP-knockout mice, with a disrupted PrP gene, show disturbances in circadian rhythm and impaired oxidative stress management [19, 20]. PrP deficient mice also show complete resistance to developing a TSE disease therefore it has been suggested that PrP\(^{C}\) plays an important role in prion propagation and TSE pathogenesis [19]. PrP\(^{Sc}\) binds to a diverse range of ligands and might be involved in neuroprotection [21]. A study published in 2010 showed a requirement of PrP\(^{Sc}\) for maintenance of the peripheral myelin sheath [22]. PrP\(^{C}\) and PrP\(^{Sc}\) share the same amino acid sequence but vary in their secondary structure and their biochemical properties [23]. PrP\(^{C}\) is a monomer composed mainly of \(\alpha\)-helices however during misfolding, PrP\(^{C}\) is converted to a \(\beta\)-sheet rich protein, PrP\(^{Sc}\), which self propagates and is prone to polymerisation with monomers of itself [24]. The conversion process is believed to occur on the plasma membrane, where PrP\(^{C}\) is functionally active, or within the endocytic pathway when PrP\(^{C}\) is shuttled away from the plasma membrane for degradation [25–27]. However, the exact mechanism of conversion and the possible involvement of any ancillary co-factors are not fully understood (table 2). Prion conversion studies in vivo and in vitro have demonstrated that the structural compatibility of PrP\(^{C}\) and PrP\(^{Sc}\) is the major determinant of the species and transmission barriers [28]. The prion species barrier or transmission barrier is the term used to characterise the extended incubation time or ablation of disease, when a species is infected by a TSE-agent originating from another species. A polymorphism at residue 129 (methionine or valine) of the prion protein has been identified to affect susceptibility to sporadic and acquired CJD [29]. So far all confirmed vCJD patients and most cases of sporadic CJD are confined to homozygotes for methionine at this residue. Heterozygosity at residue 129 appears to have a protective effect by inhibiting homologous protein-protein interactions [29].

Conformation of PrP\(^{Sc}\) might be the basis for different TSE strains

The existence of different prion strains that can be propagated in the same species has long challenged the protein-only hypothesis. Different TSE strains are characterised by variations in neuropathology, and incubation time before clinical symptoms. Since strains can be propagated in the same inbred mouse lines, strain characteristics cannot be encoded by the PrP amino acid sequence. Furthermore, no strain-specific posttranslational modifications of PrP\(^{Sc}\) have been identified. Consequently, if prions are composed

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**Figure 1A**

Schematic model of seeded polymerisation, which involves the interaction between a prion seed with PrP\(^{C}\). Partial unfolding of PrP\(^{C}\) may promote induction and propagation of PrP\(^{C}\) to PrP\(^{Sc}\). It is unknown how prion aggregates or fibrils are fragmented into reactive infectious seeds.

**Figure 1B**

Yeast and mammalian proteins with prion domains have the ability to misfold into a multitude of different conformations that encipher different biochemical and biophysical properties.
of misfolded prion protein, then different prion strains must arise from conformationally distinct amyloid forms capable of stably self-templating their specific conformations (fig. 1B). Indeed, conformationally distinct isoforms of PrPSc are reproducibly propagated upon serial passage in inbred animal lines [30, 31]. Moreover, biochemical characterisation of different prion strains demonstrate remarkable differences in the biochemical properties of PrPSc molecules, suggesting that strain specific information might indeed be “enciphered” within a specific fold of the core PrPSc aggregate [32].

Amplification of prion infectivity in vitro argues against the virion theory

Despite accumulating evidence implicating PrP as the major component of the infectious agent, the protein-only hypothesis is keenly debated by scientists who believe that the infectious agent has a pathogen coding nucleic acid [33]. However, recently designed in vitro assays such as the protein misfolding cyclic amplification (PMCA) have demonstrated that the infectious agent responsible for TSE disease could not be a virus or virion [34]. The PMCA is a procedure that is conceptually analogous to the polymerase chain reaction, where PrPSc content and prion infectivity can be drastically increased by serial cycles of polymer growth and fibril fragmentation. Minute quantities of PrPSc present in brain homogenate or blood from prion-infected animals can be used as a seed to template the conversion of substrate PrPSc [34, 35]. Sonication is used in cycles to break up aggregates, thus increasing the number of catalytic sites available for conversion [36]. This assay recapitulates key features of TSE agent replication such as the species barrier and strain characteristics, which are preserved after serial prion amplification in the PMCA. Conventional PMCA uses crude brain homogenates, therefore it cannot be ruled out that there are additional factors present in brain homogenate crucial to the conversion process. Incorporation of additional components such as poly(A)RNA and lipids into the PMCA reaction increased PrPSc and infectivity, thus indicating the possible involvement of these factors in the conversion process [37], although evidence for direct involvement of RNA in in vivo prion formation has not been shown. Alternatively, sulfated glycosaminoglycans may also serve as cellular factors necessary for in vivo and in vitro PrPSc formation [38]. Persuasive evidence that the infectious TSE agent is derived of misfolded PrP was demonstrated when recombinant PrP was converted into a β-sheet isoform that resulted in a TSE-like disease when injected into animals [39–41]. The fact that these induced prion diseases drastically differed in their clinical and pathological features from known natural prions, make it unlikely that clinical presentation in these animals was due to experimental contamination. Instead, the distinct prion phenotypes observed in vivo were likely the result of different amyloid conformers produced in vitro. However, it has been theorised that the extended incubation times of synthetic prions suggest that only a minor subfraction of PrPSc particles are infectious. Alternatively, synthetic prions are primitive surrogates of PrPSc that might evolve into infectious PrPSc or indeed may serve as a protein scaffold for de novo PrPSc formation in vivo [42].

Prions as epigenetic elements of inheritance

In 1994 the prion hypothesis was extended to include other proteins identified in lower eukaryotes that behave like prions [43] (table 1). The existence of non-Mendelian genetic elements in baker’s yeast (Saccharomyces cerevisiae) had long puzzled geneticists. These factors produced phenotypic traits that failed to segregate at mitosis suggesting that their determinants had an extrachromosomal location [43, 44]. Wickner and later Lindquist, provided evidence that these non-Mendelian heritable traits in yeast were caused by self-propagating misfolded isoforms of normal cellular proteins that when in their aggregated state show a loss-of-function phenotype [43, 45]. Similar to mammalian prions, fungal prion proteins can adopt at least two structurally distinct states (monomorphic and ordered aggregates) that differ structurally and functionally. Once misfolded, fungal prion proteins aggregate and propagate their abnormal state by templating the conversion onto the natively folded soluble monomeric isoform. Although toxic yeast prion variants might exist, yeast prions are not generally considered to be toxic to yeast and rather constitute epigenetic elements of inheritance that may play an evolutionary role in environmental adaptation [46, 47]. At least eight prions of lower eukaryotes have been identified (table 1) of which the translation termination factor Sup35 is the most studied [48]. Sup35 promotes ribosomal polypeptide chain release at the stop codon and oligomerisation of the Sup35

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<td>Timeline of important discoveries in mammalian and yeast prion research.</td>
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prion domain results in non-functional protein complexes that cause a non-sense suppression phenotype [49]. Consequently the misfolded isoform of Sup35 results in a loss of function phenotype characterised by a change in yeast metabolism. Spontaneous conversion of the Sup35 protein into its aggregated prion-like state is a very rare event and occurs at a frequency of $10^{-5} - 10^{-7}$ per cell [49]. Prion-like inheritance has also been demonstrated with the heterokaryon incompatibility factor in the filamentous fungus *Podospora anserina* [50]. Remarkably fungal prion proteins share little sequence similarity, however the prion domains of several yeast prions contain stretches rich in asparagine (Asn) and glutamine (Gln) residues that are critical for prion formation and propagation. Furthermore, randomisation of prion domains in yeast prions showed that the self-propagating capacity was retained and therefore is related to the amino acid composition rather than a specific sequence [51]. Unique prion variants in yeast, similar to that of mammalian prion strains, have been identified to be associated with specific and distinct metabolic phenotypes [52]. These heritable genetic elements may differ in their biochemical and biophysical properties but they also influence yeast-mating behaviour and differ in their transmission to daughter cells [48, 53].

Yeast prions recapitulate key characteristics of mammalian prions

Since the utilisation of yeast as a model system, our understanding of prion biogenesis has been greatly enhanced. Seminal studies in *Saccharomyces cerevisiae* demonstrated first proof of principle of the protein-only hypothesis when prion infectivity could be generated in *vitro* from pure recombinant protein [53–55]. Introduction of fibrillar recombinant fungal protein in *vitro* is sufficient to induce a prion phenotype in yeast. This observation has formed the basis for the argument that amyloid fibrils are the molecular basis of yeast prion infectivity. Furthermore, transmission experiments between *Saccharomyces cerevisiae* and *Candida albicans* demonstrated a prion transmission barrier

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<th>TSE agents</th>
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<tr>
<td>Prion Protein (PrP)</td>
<td>Sup35, Ure2, Rnq1, Het-s, Cyc8, Swi1, Mcl3, Pma1, Sfp1</td>
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<tr>
<td>GPI-anchored on plasma membrane</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Fatal transmissible spongiform encephalopathy</td>
<td>Metabolic</td>
</tr>
<tr>
<td>Plasma membrane and/or endocytic pathway</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Not well defined (aa 90-231)</td>
<td>Sup35 (N domain)</td>
</tr>
<tr>
<td>None defined</td>
<td>Chaperone Hsp104 in conjunction with Hsp70/Hsp40</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell to cell, intraspecies and interspecies (transmission barrier)</td>
<td>Yes (in cell culture)</td>
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<td>Yes</td>
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<td>Supporting evidence</td>
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Heterogeneous protein aggregates can serve as imperfect templates for prion induction

The mechanisms of prion misfolding and aggregation in spontaneous forms of TSEs remain elusive and it is the identification of factors that can influence or cross-seed the prion protein conformation that is of great scientific interest. In a study by Derkatch and colleagues to identify factors that affect the induction and propagation of prions in yeast, another cellular prion composed of the host protein Rnq1, was discovered. Rnq1 prions themselves are epigenetic elements that are required for the *de novo* formation of the Sup35 prion in yeast [58] (fig. 1C). In addition, an unbiased functional screen has identified eleven other Gln/Asn-rich proteins, which when over-expressed, can function as prion-inducing factors. The list of proteins that can promote the *de novo* conversion of Sup35 to its prion conformation can now potentially be extended to other non-prion proteins. This was directly shown when aggregates of the poly-glutamine rich region encoded by exon 1 of huntingtin acted as prion-inducing factors [60]. Interestingly, non-polyglutamine containing amyloidogenic proteins such as transthyretin, α-synuclein and synphilin cannot induce aggregation, arguing for the necessity of Gln/
Asn-rich domains for prion induction and propagation [60]. If the conformational change of a protein to a prion state can be templated by other prions, or by the over-expression of proteins with Gln/Asn-rich domains, then heterogeneous protein aggregates could catalyse the seeding of the prion conformation [61].

Involvement of the chaperone system in yeast prion replication

Prion replication and propagation crucially depend on fibril elongation and segregation, mechanisms that are not fully understood for mammalian prions. Genetic studies have shown that a chaperone network stringently controls prion propagation in yeast [62]. The central key player of this network is the protein disaggregate Hsp104 for which no mammalian homologue has been identified [63]. Hsp104 is a chaperone that cooperates with the Hsp70 machinery to regulate prion biogenesis for several yeast prions [63]. At moderate expression levels, Hsp104 assists prion propagation by fragmenting prion aggregates and thereby generating new conversion sites, whereas at high concentrations, Hsp104 disassembles fibrils into non-infectious monomers. The activity of Hsp104 is stringently controlled by molecular chaperones of the Hsp70 family and their regulating co-chaperones (fig. 1C) [62]. Other chaperone systems might also be involved in the regulation of prion biogenesis. For example, over-expression of Hsp90 significantly decreases prion induction. The involvement of chaperones in amyloid fragmentation led to the postulation that host cell factors convert an amyloid protein into an infectious aggregate [64]. The association between host cell and aggregates is likely to also influence the fate of amyloidogenic proteins in mammals in general.

Identification of other factors affecting yeast prion biogenesis

There is accumulating evidence from genetic studies that prion biogenesis is modulated by a cellular protein degradation system termed the ubiquitin/proteasome system and deficiencies in this system have been shown to increase induction of some yeast prions [65, 66]. Due to the absence of data indicating a direct ubiquitination of yeast prions prior to degradation, it is believed that the ubiquitin/proteasome system instead influences prion biogenesis by regulating ancillary factors. A study by Chernova and colleagues described the regulation of prion biogenesis by ubiquitination and proteasomal degradation of the prion-inducing factor, Lsb2 [67]. Inhibition of its ubiquitination and subsequent degradation resulted in constantly elevated levels of this factor, which in turn increased levels of prion induction. A number of recent studies have been published describing the association between prion biogenesis and the actin cytoskeleton. The yeast prion Sup35 interacts with several proteins of the actin cytoskeleton and alterations of actin polymerisation decrease Sup35 prion induction [68]. In addition, the association of the previously described prion-inducing factor, Lsb2, with the actin cytoskeleton, is required for its prion-inducing function [67]. A different experimental approach was by proteomic analysis, which identified several proteins that associated with yeast prions and may function as yeast prion modifiers [69]. In conclusion, studies on yeast prions have shown a direct regulatory process by the host cell on prion induction and propagation. Therefore, the identification of novel factors that mediate spontaneous induction and propagation of prions in yeast will likely also facilitate the discovery of therapeutic inter-

Functional amyloid and beneficial prions

Historically, amyloid has been defined as β-sheet rich extracellular proteinaceous deposits associated with neurodegenerative disorders. Today, amyloid is often defined biophysically as fibrillar ordered protein-aggregates exhibiting characteristic cross-β-sheet x-ray diffraction patterns. Our view of amyloid as disease-associated protein aggregates has drastically changed to incorporate the findings that there are amyloid fibrils performing beneficial functions in prokaryotes as well as lower eukaryotes and even mammals [70, 71]. Cellular processes appear to tightly control functional amyloid formation to prevent uncontrolled spreading and subsequent cell damage. Amyloid has been shown to be an abundant component of natural biofilms formed by a wide range of bacteria. Both silkmoth choriom proteins and spider silk are composed of amyloid fibrils [72, 73]. Other functional amyloid has been proposed in important biological processes; the aggregation of the protein CPEB to amyloid may contribute to the basis for long-term memory in sensory neurons of Aplysia [74]. In mammals, the Psen17 protein in its amyloid state is a functional component of melanosomes [75]. In addi-
tion, it has been shown that even certain hormone peptides form amyloid-like aggregates when packaged into secretory granules [76]. In this case, the obvious advantage for aggregation is that the amyloid conformation allows for a denser packaging of the peptide hormone for future demand. Clearly, these studies have already uncovered that amyloid is much more abundant than previously anticipated.

The identification of the heterokaryon incompatibility factor Ht-s in Podospora anserina with gain-of-function associated with its amyloid-like state goes beyond the concept of physiological amyloid, showing that some amyloids can even rapidly spread and propagate their aggregated states in a prion-like manner [77]. This has raised the question, how common is the prion phenomenon and could there be more beneficial prions? Proteins with prion functionality are continuously being characterised in yeast and genome wide searches in lower and higher eukaryotes, including mammals, have identified many proteins that may have the potential to act like prions [78]. Clearly, cellular mechanisms exist in the mammalian cytosol that supports prion-like inheritance [79]. Thus, it is tempting to speculate that additional prions will also be discovered in mammals.

Concluding remarks

Over the past few years important work in the model organism, Saccharomyces cerevisiae, has shed new light on the molecular mechanisms that govern prion biogenesis. Research on yeast prions provided first evidence that protein polymers can act as infectious entities and can faithfully store and propagate strain information. This work also illustrated the crucial role of host cell factors for spontaneous prion induction and fragmentation. Whilst these factors may be unlikely to be identical in the biogenesis of the TSE agent in mammals (primarily because PrP conversion in mammals is either occurring extracellularly on the plasma membrane or within endocytic vesicles), they highlight that the intimate interaction of amyloidogenic protein aggregates and the host cell is a critical determinant in the adaptation from amyloid to an infectious protein entity. These data become even more valuable as recent studies suggested that several amyloids associated with some of the most prevalent human neurodegenerative diseases might spread in a prion-like manner [80–82]. Therefore, the identification of prion regulatory factors in yeast could facilitate in the characterisation of common mechanisms that underlie the formation and propagation of disease-related amyloid. Studies on prions and amyloid in lower eukaryotes have also brought up the intriguing concept that conversion to the amyloid or prion conformation might not necessarily be detrimental to the host. With the benefit of a tractable model in yeast, it will be the challenge of future research to identify these proteins and to understand the physiological relevance of their amyloid-like conformations in health and disease.

Acknowledgements: We thank Sybille Krauss and Walker Jackson for critical reading of the manuscript.

Authors’ contributions: * These authors contributed equally to the work

Funding / potential competing interests: This work was supported by the DFG grants IV 1277/1-3 and SFB 569 B14.

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Schematic model of seeded polymerisation, which involves the interaction between a prion seed with PrP\textsuperscript{C}. Partial unfolding of PrP\textsuperscript{C} may promote induction and propagation of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}. It is unknown how prion aggregates or fibrils are fragmented into reactive infectious seeds.

Yeast and mammalian proteins with prion domains have the ability to misfold into a multitude of different conformations that encipher different biochemical and biophysical properties.
The induction, propagation and fragmentation of mammalian and yeast prions are compared. Partial unfolding of proteins with prion domains and/or the presence of other cellular factors may influence susceptibility to misfolding. (i) In the presence of a prion seed, PrP<sup>Sc</sup> is converted to PrP<sup>Sc</sup> thereby elongating the PrP<sup>Sc</sup> fibril. Conversion is likely to be aided by the presence of molecules such as poly(A)RNA, lipid and/or other co-factors and aggregates are fragmented by an as yet unidentified fragmentation process. (ii) Induction of yeast prions depends on interaction with actin cytoskeleton and/or cross-seeding by other yeast prions or yeast proteins with Gln/Asn-rich domains. Propagation and fragmentation of yeast prion aggregates is performed by the yeast protein chaperones Hsp104 and Hsp70/ Hsp40.
Table 2
Timeline of important discoveries in mammalian and yeast prion research.