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What Makes a Prion: Infectious Proteins From Animals to Yeast

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Running title: Infectious Proteins from Animals to Yeast

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2 Tables, 4 Figures

Abbreviations: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; CJD, Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; MBM, meat and bone meal; ALS, amyotrophic lateral sclerosis; FTLD, frontotemporal lobar degeneration; MSA, multiple system atrophy; TMV, tobacco mosaic virus; PrP, prion protein; PFD, prion-forming domain; PrLD, prion-like domain; ND, nucleation domain; ORD, oligopeptide repeat domain; PrP, mammalian prion protein; HMM, hidden Markov model; GFP, green fluorescent protein; ORF, open reading frame.

Abstract (100-150 words, max 250)

While philosophers in ancient times had many ideas for the cause of contagion, the modern study of infective agents began with Fracastoro's 1546 proposal that invisible "spores" spread infectious disease. However, firm categorization of the pathogens of the natural world would need to await a mature germ theory that would not arise for three hundred years. In the 19th century, the earliest pathogens described were bacteria and other cellular microbes. By the close of that century, the work of Ivanovsky and Beijerinck introduced the concept of a virus, an infective particle smaller than any known cell. Extending into the early-mid 20th century there was an explosive growth in pathogenic microbiology, with a cellular or viral cause identified for nearly every transmissible disease. A few occult pathogens remained to be discovered, including the infectious proteins (prions) proposed by Prusiner in 1982. This review discusses the prions identified in mammals, yeasts, and other organisms, focusing on the amyloid-based prions. I discuss the essential biochemical properties of these agents and the application of this knowledge to diseases of protein misfolding and aggregation, as well as the utility of yeast as a model organism to study prion and amyloid proteins that affect human and animal health. Further, I summarize the ideas emerging out of these studies that the prion concept may go beyond proteinaceous infectious particles and that prions may be a subset of proteins having general nucleating or seeding functions involved in non-infectious as well as infectious pathogenic protein aggregation.

Key words: prion, amyloid, PrP, human, yeast, Sup35, [*PSI*$^{+}$], Ure2, [URE3], nucleation, propagation, maintenance, composition, amino acids, bioinformatics, prionoid, quasi-prion

1. Introduction

As long as there have been humans, curing and preventing illness in humankind has been a goal that crosses all cultural and geographic boundaries. Key to any real understanding of how to heal the sick was careful study of illness, identification of true causes of diverse types of sickness, and experiments to assess methods of cure and prevention. This article explores the historical development of infectious disease etiology (section 2) culminating in the proposal of a purely protein-based infectious agent, the prion. Scientific evidence for the existence of infectious prions in animals and in yeasts and other species is presented in section 3. While a subset of proteins were identified with this unusual pathogenicity and transmissibility, the essential question of why only some proteins displayed this behavior was the next big question, addressed in section 4. Some answers of what makes a protein a prion grew out of basic structural characterization of prions, examining their amyloid structure, and further experiments in animals and yeasts have begun to fine-tune that understanding. Finally, this growing understanding of prions has had implications for non-infectious protein aggregation diseases in humans and animals and has led to an enlargement of the prion concept, discussed in section 5.

2. Pathogens and the Emergence of the Prion Hypothesis

2.1 The causative agents of infectious disease

Diseases of antiquity such as leprosy and plague left indelible marks on cultures and civilizations but also had no known and agreed-upon cause. Some blamed supernatural forces, others vapors and miasmas, and still others diet, living conditions, and

atmospheric climate. The ancient Greek physician Galen, working in the 2nd century CE from the medical principles of Hippocrates and others, was the primary proponent of the idea of diseases caused by miasma ("pollution") or poor quality air. In 1546, Girolamo Fracastoro, the eminent Venetian physician, published his work *De Contagione et Contagiosis Morbis* promulgating the idea of "spores," directly transmitted (*contagion*) and also distantly transmitted, and fomites 'not themselves corrupt' indirectly spreading these seeds of disease. This work was published during the time he was serving as the elected physician of the Council of Trent and proved to be an influential counterpoint to the prevailing notion of miasmas. However, Galen's miasma theory of disease would not be fully supplanted in the minds of physicians and scientists until the last years of the 19th century with the advent of the germ theory of disease (Table 1).

2.2 Cellular causes of infectious diseases

A medieval Dutch draper who wanted to see his threads better, Antonie van Leeuwenhoek, became the celebrated lens and microscope maker that introduced the world to the first observations of microscopic organisms. Beginning in 1673, van Leeuwenhoek's 190 letters to the Royal Society described observations of the first cells that he termed *animalculum* ('very small animals'). In the course of his work, van Leeuwenhoek noted not only the first unicellular organisms (protists) but also the first bacteria and subcellular structures. The English scientist Robert Hooke coined the term *cell* in his 1665 book *Micrographia* to describe the individual compartments in cork and living plants that were analogous to the *animalcules* of van Leeuwenhoek.

Although microscopic cells and microbes were known from the 17th century, for nearly two hundred years after van Leeuwenhoek and Hooke doctors and scientists saw no connection between the cellular microbes and disease, even in some cases postulating that organisms found in diseased tissues were the effect, rather than the cause, of injury. A 'germ theory' arose in the 19th century, connecting the presence of infectious organisms with disease. Agostino Bassi (1838, silkworm disease) gained rapid acceptance for his work but Ignaz Semmelweis (1847-1861, childbed or puerperal fever) met with substantial resistance for a germ theory of disease.

The French chemist Louis Pasteur firmly established the germ theory of disease with his experiments demonstrating a microbial cause for fermentation, disproving spontaneous generation, developing 'pasteurization,' and linking particular silkworm diseases to microbes (1857-1870). German scientist Ferdinand Cohn soon formally described and classified the *Bacteria* (1875). Visiting Cohn at Breslau, physician Robert Koch demonstrated the use of pure cultures of anthrax bacilli to cause the illness in previously healthy animals (1876 with refinements continuing in later years). While developing his famous postulates for connecting specific microorganisms with specific diseases, Koch in the 1880s made several other connections between disease-causing or pathogenic organisms and their specific organic diseases, notably cholera and tuberculosis. Many other scientists and physicians contributed their observations to the growing body of evidence that supported the germ theory of disease.

2.3 Non-cellular causes of disease in animals

Building on the work of Pasteur, Koch, and others in the mid-late 19th century, the microbiological agents responsible for the great diseases of antiquity were, one after another, systematically identified. As described, the first pathogenic agents identified were those in which the organisms in question could be readily observed under the microscope, such as Pasteur's discovery of a microsporidian parasite as the cause of the pébrine disease of silkworms and Koch's discovery of the bacterium *Bacillus anthracis* as the cause of anthrax.

However, some diseases stymied the efforts of even the giants of the new fields of bacteriology and microbiology. Although Pasteur successfully developed a rabies vaccine in 1886, he could not identify the causative agent, speculating that it was too small to be visible through the use of the microscope. Another French microbiologist, Charles Chamberland, developed a special porcelain filter that excluded anything as large as the known bacteria (1884). The Chamberland Filter proved important for extending the germ theory of disease beyond the cellular parasites, protists, and bacteria. Russian scientist Dmitri Ivanovsky used a Chamberland Filter to remove bacteria and isolate the tobacco mosaic virus (1892) although it was not initially perceived to be anything other than a bacterial toxin. The Dutch microbiologist Martinus Beijerinck in 1898 realized that Ivanovsky's filtrate actually contained a new infectious agent that he referred to both as a *contagium vivum fluidum* ('living fluid germ') and as a *virus* ('slimy poison liquid'). In the same year, Friedrich Loeffler and Paul Frosch discovered the first animal virus (aphthovirus for foot-and-mouth disease) using a similar filter.

The composition of viruses was not immediately understood. American virologist Wendell Stanley, working with Ivanovsky's filtered agent, now known as tobacco mosaic virus (TMV), successfully crystallized it, proving it was not a liquid as Beijerinck has proposed. However, Stanley initially believed that TMV contained only protein and only later realized the concomitant presence of a nucleic acid (Stanley 1935; Cohen, SS 1942). The scientific community had not yet firmly settled on nucleic acid as the particle of heredity by this time, but evidence was accumulating.

Since Friedrich Miescher's 1869 discovery of the *nuclein* or nucleic acid found in nuclei of eukaryotic cells, scientists had been probing its structure. Phoebus Levene's 1919 tetranucleotide hypothesis of nucleic acid structure (Levene 1919) held sway in the scientific community for decades, suggesting nucleic acid would be a poor informational molecule and that therefore protein would be a superior basis for the particles of heredity. When Frederick Griffith's 1928 pneumococcal 'transforming principle' (molecule of heredity) (Griffith 1928) was proven to be nucleic acid (Avery et al. 1944), the composition and structure of viral genetic information also became a point of intense interest. It was Alfred Hershey and Martha Chase, working with bacteriophage (bacterial virus) T2, who demonstrated that the nucleic acid portion of the virus was its hereditary material as well (Hershey & Chase 1952).

By this time, a host of viruses had been identified as the causative agents of plant and animal diseases, complementing the many cellular pathogens identified in the 19th and early 20th centuries. By the mid-20th century, the majority of the pathogenic agents causing known infectious diseases had been identified (Brachman 2003). All of these agents were cellular or viral in nature.

2.4 Unusual disease traits in animals

Despite success with identifying many cellular and viral pathogens, the cause of a few rare diseases remained stubbornly difficult to pinpoint.

One of these diseases was a condition known as scrapie observed in Merino sheep in Spain in 1732 (Table 2, top). This disease, in which sheep obsessively scrape themselves against trees, fence posts, and other obstacles, also manifests a variety of symptoms affecting the nervous system: altered gait, lip smacking, and convulsions. Although clearly infectious within flocks, long and variable incubation periods made determination of etiology difficult. No virus or cellular cause had been identified as a cause of scrapie, but it had been hypothesized that the disease was caused by a 'slow virus,' an exceptionally slow-to-propagate virus with a long incubation period (Cuille & Chelle 1938a; Sigurðsson 1954).

Human diseases of unknown etiology were found with similarities to scrapie (Table 2, bottom). A human neurological disorder that would come to be known as Creutzfeldt-Jakob disease (CJD) was identified in 1920 (Creutzfeldt 1920; Jakob 1921). Another human disease found among the Fore tribe of Papua New Guinea, called kuru or the 'laughing disease,' was brought to the attention of the scientific community in 1959 (Gajdusek & Zigas 1959; Klatzo et al. 1959). Immediately, the similarities in these diseases were noted (Hadlow 1959; Klatzo et al. 1959) and it was postulated that all of them were infectious (like scrapie) and due to a slow virus. Later experiments proved their transmissible nature and these diseases came to be known as transmissible

194 spongiform encephalopathies (TSEs) on the basis of their essential neuroanatomic effect
195 of producing tiny holes in the brain cortex of affected individuals (Fig. 1).
196

197 ## 2.5 Non-Mendelian inheritance of characters in the baker's yeast

198 In 1965, yeast geneticist Brian Cox traced and described an unusual trait he called [ψ^+]
199 (now written as [*PSI*$^+$]) in the baker's yeast *Saccharomyces cerevisiae.* The [*PSI*$^+$] trait
200 was a suppressor of a super-suppressor of stop codons, a gene now known as *SUP35*.
201 What made the trait more puzzling was that in Cox's meticulous studies of inheritance,
202 [*PSI*$^+$] did not obey Mendelian principles of inheritance (Cox 1965; reviewed in Tuite et
203 al. 2015). Cox identified (correctly) what he referred to as a 'self-replicating particle' in
204 the cytoplasm that was involved in the inheritance of the trait. In yeast, there were three
205 known principle cytoplasmic components that were inherited: mitochondrial DNA, yeast
206 killer dsRNA plasmids, and 2-micron circle plasmids. The [*PSI*$^+$] trait was none of these,
207 although its identity would remain a mystery for almost 30 years.

208 Another strangely inherited trait in yeast was identified by Francois Lacroute in 1971
209 (Lacroute 1971). In this case the gene involved was called *URE2* and the trait [URE3].
210 Lacroute hypothesized that the trait was mitochondrially inherited, although several
211 features would have been very unusual for a mitochondrial trait. Lacroute also proposed
212 an alternative to that idea, proposing that [URE3] was a 'non-mitochondrial cytoplasmic
213 replicon' of unknown nature (Lacroute 1971). Akin to [*PSI*$^+$], the biochemical and
214 genetic basis of [URE3] was not understood until the prion hypothesis had been in
215 formulated. Connection of these traits to the prion hypothesis (discussed next) will be

described in section 3.7 below.

2.6 The prion hypothesis

In the animal TSEs, the hypothesis of a slow virus etiology was widely accepted, but data began to accumulate that put that etiology into question. CJD in humans was clearly hereditary. The scrapie agent was not inactivated by formalin or by UV radiation, which both inactivated known viruses (Alper et al. 1967; Pattison & Jones 1967). Decades of struggle to find any nucleic acid in the scrapie agent continued to prove fruitless and several investigators suspected a purely proteinaceous infective nature for scrapie (Griffith 1967; Hunter et al. 1969; Prusiner, Hadlow, Garfin, et al. 1978; Prusiner, Hadlow, Eklund, et al. 1978; Prusiner, Groth, Cochran, McKinley, et al. 1980; Prusiner, Groth, Cochran, Masiarz, et al. 1980; Hadlow et al. 1980; Prusiner et al. 1981; Cho 1980; Merz et al. 1983).

Despite the lack of evidence for nucleic acid playing a role in transmission for the TSEs, the scientists working in the field still had a healthy regard for the Central Dogma and were not ready to assume a protein-only inheritance for these diseases. However, one scientist, Stanley Prusiner, was willing to push ahead with a formal hypothesis of a fully protein infective agent, something he called the 'proteinaceous infectious particle' or 'prion' (Prusiner 1982). This bold hypothesis, for which Prusiner would be awarded the Nobel Prize in Physiology or Medicine in 1997, was not proven overnight, and many lines of evidence were required to convince a skeptical scientific community. This hypothesis would later be more widely applied to the inheritance of the unusual non-Mendelian characters in yeast and what was learned in the study of prion diseases would

239 prove applicable to the more general problem of human protein-misfolding diseases that
240 were of a non-infectious nature as well.
241

242 3. Evidence Found: Identification of Animal, Yeast, and
243 Other Prions

244 3.1 Scrapie in sheep and goats

245 TSEs have been found in a number of mammals, including humans (Table 2) with the
246 longest studied being scrapie. Sheep and goats affected with the neurological pathology
247 of scrapie had been the subject of scientific investigation for centuries, with the first
248 verified report published in Germany in 1750 (Leopoldt 1750) although cases were cited
249 in other reports going back to 1732 in Spain and in England. Leopoldt's initial report
250 postulates an infectious cause for scrapie although other scientists would debate whether
251 hereditary or other causes were more likely for many years to come (reviewed in
252 Schneider et al. 2008). Experiments to prove transmissibility were undertaken many
253 times, but had various deficiencies leading to continued disagreement. Finally, beginning
254 in 1936, Cuille and Chelle proved transmissibility by inoculating healthy animals with
255 material from the central nervous systems of sick animals (Cuille & Chelle 1936; Cuille
256 & Chelle 1938a; Cuille & Chelle 1938b; Cuille & Chelle 1938c; Cuille & Chelle 1939).
257 Small wild sheep called mouflons are also susceptible to scrapie (J. Wood et al. 1992), as
258 are goats (Cuille & Chelle 1939; J. N. Wood et al. 1992).

259 Cuille and Chelle proposed a viral etiology for scrapie in their 1930s research, although
260 other causes were still postulated by others. A particular designation as a 'slow virus'
261 disease (Sigurðsson 1954) became the common way to group this disease with CJD and
262 Kuru as they were discovered. As mentioned above, a protein-only transmission was also
263 proposed by Griffith but did not immediately attract the support of the scrapie research
264 community (Griffith 1967). One difficulty in conducting this research was the long
265 incubation in sheep, which was overcome by conducting experiments in mice (Chandler
266 1961). Although mice remained a workhorse in studying scrapie for decades, a later
267 hamster model was also developed which dropped the incubation period from years in
268 sheep to 150 days in mice to 60 days in hamsters (Kimberlin & Walker 1977).

269 The prion protein was identified and called PrP, with the gene being called *Prnp* in sheep
270 and goats. Two forms were described: PrP^{Sc} (scrapie form) and PrP^{C} (cellular normal
271 form). Many strains of scrapie were identified, mutations in the genes were identified,
272 and it was found that some strains/mutations delayed onset of disease and others
273 shortened the time to disease progression.

274 Scrapie modes of transmission have been debated for many years. Although
275 experimental transmission can take several forms, the natural transmission of scrapie
276 horizontally between individuals occurs through direct contact between animals and
277 through contact with environmental contamination (reviewed in Schneider et al. 2008).
278 Scrapie is predominantly acquired through the oral route and the placenta and amniotic
279 fluid are the most common sources of oral infection, although fetal parts, feces, and milk
280 have all shown infectivity (see Schneider et al. 2008).
281

3.2 Bovine spongiform encephalopathy

With the substantial neuropathological understanding of scrapie going back decades, veterinarians and scientists in the United Kingdom quickly noticed the arrival of a new, related disease. Bovine spongiform encephalopathy (BSE) in cattle was identified in 1987 (Wells et al. 1987). BSE was noted for the classic neurological symptoms typical of spongiform encephalopathies: ataxia (contributing to 'downer cattle' that cannot stand well), behavioral changes, anorexia, and death. The practice of using rendered meat and bone meal (MBM) product (which contains nervous tissue) from sheep and cattle to increase protein in animal feed was immediately suspected as a potential epidemiological cause of the BSE outbreak (Taylor 1989; Matthews 1990) and UK and other government inquiries agreed with that stance, leading to changes in feeding practices across the globe. It is still debated whether BSE may have arisen from sporadic BSE entering the MBM food chain or whether it may have been scrapie in slaughtered sheep in the MBM (with a subsequent rare evasion of the species barrier) that led to the widespread BSE outbreak in the United Kingdom. It was quickly recognized, however, that since a scrapie origin to the BSE outbreak was plausible, the possibility that BSE might also cross the species barrier into humans was equally plausible (Taylor 1989; Matthews 1990). This prediction proved prescient, with the discovery of an unusual cluster of younger Creutzfeldt-Jakob patients ("variant" CJD) in the United Kingdom only a few years later in 1996 (see the next section for a fuller description).

3.3 Kuru, CJD, other prion diseases in humans

The first description of a human TSE disease (Table 2, bottom) was Creutzfeldt-Jakob disease in 1920-21 (Creutzfeldt 1920; Jakob 1921). This rare, neurodegenerative disease (CJD) was characterized in people by loss of memory and judgment and increasing dementia, concomitant with loss of muscular coordination, significant personality changes, and impaired vision. The proximate cause of these neurological deficits was death of neurons (as seen in MRI, Fig. 1A) and holes in brain tissue with concomitant buildup of plaques (as shown in histologic section, Fig. 1B). CJD was found to occur in families but most cases were not associated with heredity and were termed sporadic CJD (sCJD). sCJD is the most common human prion disease with ~85% of all cases, with the balance made up of familial CJD and other diseases (Prusiner 1989).

Kuru (Gajdusek & Zigas 1959; Klatzo et al. 1959) bore many of the same neurological features as CJD and scrapie when it was identified among the Fore people of the Eastern Highlands of Papua New Guinea. Originating from a Fore word meaning "to shake," kuru was also known among the Fore as the 'laughing sickness.' The Fore engaged in a practice of mortuary or funerary cannibalism wherein the internal organs, including the brain, of the dead would be consumed by living relatives for spiritual purposes (Alpers 1968). When Australian colonial administrators and Christian missionaries suppressed the practice of cannibalism, the epidemic levels of kuru observed in the 1950s rapidly declined, although because of the long and variable incubation period seen in many TSEs the last sufferer of kuru is reported to have died in 2005 (Alpers 2008; Lindenbaum 2008; Anon 2009).

Beginning in the 1990s, it was recognized that human disease caused by prions went beyond the sporadic or familial forms of CJD and the exotic and largely extinct kuru. Variant CJD (vCJD) was noted in the United Kingdom in 1996, with features consistent with a CJD diagnosis, but an earlier average age of onset (Will et al. 1996). It was rapidly shown that the cause of the vCJD outbreak was consumption of food products from cattle infected with the BSE agent (Bruce et al. 1997).

Iatrogenic CJD (iCJD) has been recognized since the 1980s. In this form of CJD, improperly disinfected medical equipment, especially instruments used in brain surgeries, and also improperly prepared medicines, *e.g.,* human growth hormone, have resulted in cases of CJD (Rappaport 1987; Marzewski et al. 1988; Mocsny 1991).

Finally, a few other distinctive human diseases with a prion basis are recognized. Fatal insomnia is a disease characterized by thalamic degeneration, progressive loss of neurological characteristics required for sleep, motor abnormalities, and hyperactivation of the autonomic nervous system (Lugaresi et al. 1986). First identified was a familial form of this disorder referred to as fatal familial insomnia (FFI) (Lugaresi et al. 1986) although later work found evidence of sporadic cases (sFI) as well (Montagna et al. 2003; Barash 2009; Moody et al. 2011). Gerstmann–Sträussler–Scheinker (GSS) syndrome (reviewed in Liberski 2012) is a very rare hereditary disease inherited in autosomal dominant fashion originally noted over 100 years ago in Austria (Dimitz 1913) and more fully described in the 1920s and 1930s (Gerstmann 1928; Gerstmann et al. 1936). GSS features dysarthria, ataxia, and progressive dementia, and its causative mutations in the human *PRNP* gene were identified in 1989 (Hsiao et al. 1989). The disease effects were experimentally recreated in mice shortly thereafter (Hsiao et al. 1990). Other variations

348 in *PRNP* associated with disease in human families have been reported in unrelated
349 groups around the world (*e.g.,* Hsiao et al. 1991; Dlouhy et al. 1992).
350

351 ## 3.4 Prion diseases in other mammals

352 Other mammalian prion diseases have been described (Table 2, top) (reviewed in
353 Greenlee & Greenlee 2015). An infectious encephalopathy affecting ranched mink
354 appeared as early as 1947 in the United States with a formal description in 1965
355 (Hartsough & Burger 1965; Burger & Hartsough 1965; Marsh & Hanson 1969; Barlow
356 1972). A disease of abnormal behavior, severe anorexia, and rapid death was observed
357 1967-1979 in cervids (elk and deer) in Colorado and Wyoming (Williams & Young
358 1980). Because of the substantial wasting caused by the anorexia in these animals, it was
359 named Chronic Wasting Disease (CWD). Despite its different name, it was immediately
360 recognized, based on distinctive histopathology, as a spongiform encephalopathy in the
361 same line as scrapie. Feline spongiform encephalopathy (FSE) was identified in
362 domestic cats (Wyatt et al. 1991; Pearson et al. 1991; Pearson et al. 1992) and later in
363 many wild cats including lions, puma, ocelot, and cheetah (*e.g.,* Eiden et al. 2010). An
364 abstract from the Prion 2012 meeting in Amsterdam reported the case of a 9 week old
365 Rottweiler with canine spongiform encephalopathy (David & Tayebi 2012). However,
366 no further reports on canine spongiform encephalopathy have been published. Even
367 though the list of species with documented cases (Table 2) is small, it remains likely that
368 yet-undiscovered spongiform encephalopathies exist in all mammals.
369

3.5 Prions in other eukaryotes

Prion-based TSEs have only been reported in mammals. However, homologues of the PrP-encoding gene have been identified in birds, reptiles, amphibians, and fish (reviewed in Schätzl 2007 and Málaga-Trillo et al. 2011). It is unknown whether the variant PrP sequences in these species (which have several divergent features depending on taxonomic grouping) can form *bona fide* prions, amyloids, or whether TSE-like disease is present in these animals.

A protein with prion characteristics, when expressed in the yeast system, was also recently found in *Arabidopsis*, making it the first potential plant prion-like protein (Chakrabortee et al. 2016; discussed in Chernoff 2016).

3.6 Evidence in support of the prion hypothesis in mammalian disease

The proposal of a fully proteinaceous infectious agent and the coining of the term prion for that agent (Prusiner 1982) did not coincide with irrefutable proof of the prion hypothesis, and certainly did not immediately satisfy all criticisms with the hypothesis. Instead, the formal statement of the prion hypothesis as the causative agent of scrapie built upon the steady framework of evidence from earlier studies (Griffith 1967; Hunter et al. 1969; Prusiner, Hadlow, Garfin, et al. 1978; Prusiner, Hadlow, Eklund, et al. 1978; Prusiner, Groth, Cochran, McKinley, et al. 1980; Prusiner, Groth, Cochran, Masiarz, et al. 1980; Hadlow et al. 1980; Prusiner et al. 1981; Cho 1980; Merz et al. 1983) and provided a scaffold upon which to place further empirical data to support or refute it. Some of the major lines of support are provided here, although other texts provide a more

complete picture of the supporting arguments (Hörnlimann & Riesner 2007; Colby & Prusiner 2011b; Zabel & Reid 2015)

The laboratories of Charles Weissmann, Stanley Prusiner, and Leroy Hood, together published the identification of the gene responsible for scrapie, which encoded a protein in sheep for which several normal functions have since been determined, but no single well-determined role has been pinpointed. The gene, *Prnp* in animals and *PRNP* in humans, encoded the PrP (prion) protein (Oesch et al. 1985). The *Prnp* gene in mice was found to be co-located with a previously identified marker of mouse scrapie called *Sinc* (Dickinson et al. 1968), which provided evidence that a normal cellular (non-viral) gene locus was associated with the disease protein (Carlson et al. 1986; Hunter et al. 1987; Carlson et al. 1988). Mice that were devoid of the PrP gene proved to be resistant to scrapie (Büeler et al. 1993). Mice that were modified to express their *Prnp* gene with the mutation corresponding to human FFI were spontaneously stricken with prion disease (Jackson et al. 2009). Prions can be made in bacteria and cause disease in mice (Legname et al. 2004). Reconstitution of the prion using a cyclic amplification technique was possible with both partially purified substrates (Deleault et al. 2005) and with infectious particles created *in vitro* (Barria et al. 2009). Further studies building on this theme show that it is possible to make recombinant infectious particles *de novo* in bacteria and without amplification in a clean laboratory that has never seen prions (Zhang et al. 2013).

The prion hypothesis holds that a natively folded cellular protein can assume an abnormal, infectious and pathological shape that can be propagated between cells and between organisms without the need for any nucleic acid or viral structures. Although

some scientists remain doubtful (Manuelidis 2007; Bastian et al. 2007; Manuelidis et al. 2009; Somerville & Gentles 2011; Manuelidis 2013), with the evidence above and other lines of evidence, most scientists are now convinced of the validity of the prion hypothesis in mammals (and, as seen below, in yeast).

3.7 Reed Wickner's keen observations in yeast

The yeast traits (discussed in section 2.5 above) that resulted from Cox and Lacroute's mysterious non-mitochondrial cytoplasmic particles in the baker's yeast *Saccharomyces cerevisiae* (Cox 1965; Lacroute 1971) had long been on the mind of Reed Wickner, yeast geneticist and virologist. He began studies in 1989 (Wickner 2012) to see if Prusiner's proposed framework of protein-only inheritance (Prusiner 1982) could be applied to the [URE3] trait.

In 1994, Reed Wickner published this work of careful and keen observation, showing that [URE3] trait resulted from a heritable conformation of the Ure2 protein, wherein it took on a prion form that was passed to daughter cells (Wickner 1994). This elegant hypothesis accounted for all of the unusual features of the non-Mendelian cytoplasmic inheritance of [URE3] that had vexed scientists for 30 years and immediately also suggested a mechanism for the inheritance of [*PSI*$^{+}$] as well (Wickner 1994; reviewed in Tuite et al. 2015). [*PSI*$^{+}$] proved to be a heritable prion state of the Sup35 protein in yeast (Doel et al. 1994; Ter-Avanesyan et al. 1994; Patino et al. 1996; Paushkin et al. 1996).

In establishing the prion hypothesis for yeast proteins, Wickner had laid out three genetic criteria for a prion that should readily distinguish them from agents containing nucleic acid, such as viruses (Wickner 1994; Wickner 2012): (a) the infection should be curable but reversible, (b) the overproduction of the relevant cellular gene should increase the frequency of prion formation, and (c) the prion-positive phenotype, inactivating a cellular protein's normal function, should match that of the loss-of-function mutant form of the same protein. All three of these criteria are met in [URE3] and [*PSI*$^{+}$], where, first, low concentrations of guanidine HCl can cure prions (Tuite et al. 1981; Lund & Cox 1981; Ferreira et al. 2001), but prions can then arise *de novo* in cured strains because the normal protein is still present. (Viruses would need to have nucleic acid reintroduced from outside the cell.) Secondly, overproduction of prion proteins increases the concentration of these proteins in the cell resulting in more prion formation (Chernoff et al. 1993; Wickner 1994; Derkatch et al. 1996), presumably due to an increase in the probability of the misfolding event that initiates prion or oligomer formation. Finally, the *URE2* and *SUP35* genes, respectively, are necessary for the formation of the [URE3] and [*PSI*$^{+}$] prions, and the prion phenotype is the same as that of loss-of-function mutations for each gene (Aigle & Lacroute 1975; Cox et al. 1988; Wickner 1994).

With these criteria satisfied, further characterization of the nature of these prion proteins could begin. Through the work of Wickner's laboratory and the labs of Michael Ter-Avanesyan, Susan Lindquist, and Susan Liebman, and others, [URE3] and [*PSI*$^{+}$] began to reveal their secrets. Comparisons with the structures of animal prions would show many commonalities.

3.8 Other fungal and invertebrate prions

Although they are not further discussed in this review, prions in other fungi and invertebrates have also been identified, which differ in some way from the known yeast and animal prions. For example, there is another fungal prion that differs somewhat in structure from the well-characterized yeast prions: [Het-s] the prion form of the HET-s protein in *Podospora anserina* (Coustou et al. 1997; Baxa et al. 2007; Mathur et al. 2011; Wan & Stubbs 2014; Wickner et al. 2016). Enzymatic and non-amyloid prions have also been identified, *e.g.,* the yeast protease B (Jones 1991; Roberts & Wickner 2003) and the poly-A binding protein CPEB in *Aplysia californica* (Si, Lindquist, et al. 2003; Si, Giustetto, et al. 2003; Si et al. 2010; Stephan et al. 2015; Si & Kandel 2016).

4. What Makes a Prion: Features that Define Prions

4.1 Defining features of prions

In the course of finding evidence for the prion hypothesis in animals and fungi (see section 3 above), many other characteristics about their biochemical and biophysical nature were also noted.

The primary physical characteristic of prions found in prion diseases is that these diseases exhibit amyloid deposits in nervous tissue (detailed below). In the course of early studies of these diseases, the amyloid deposits were found to be stainable with agents such as Congo red. After the identity of amyloid as protein rather than either carbohydrate or

lipid, amyloid proteins were also found to be insoluble, protease and detergent resistant, beta-sheet rich, and prone to assemble into aggregate and fibril structures.

In this section, I detail the work that uncovered the overall amyloid structures of the animal (section 4.2) and yeast (section 4.3) prions. Knowledge of the essential structural and functional nature of prions (PrP and the yeast prions, chiefly) has logically led to the search for other prions in mammals and in yeasts (section 4.4), although the success rate for finding new prions has been much greater in yeast. Other characteristics that define prions have also been noted over years of study (section 4.5) and these characteristics are leading to insight into prion, amyloid, and similar diseases and their pathophysiologies.

4.2 Structural features of animal prions

Animal prions are characterized by certain structural and biochemical features. The well-characterized mammalian PrP prion is known to form amyloid fibrils. Amyloids (misidentified by Rudolf Vircow in 1854 as related to starch—*amylum*—because amyloid is stained by iodine like starch) were found in nervous tissue and associated with all of the prion diseases above as well as with other amyloidoses including Alzheimer's disease (Sipe & Cohen 2000). Amyloids were found to be different from starch under light microscopy on the basis of a green/yellow/orange birefringence when stained with Congo red dye and illuminated under polarized light (Howie 2015). In 1959 the first electron micrographs of amyloids showed fibrils of 80-100 Å in width and of variable length (Sipe & Cohen 2000). Amyloids were resistant to protease treatment (McKinley et al. 1983;

500 Oesch et al. 1985; Manuelidis et al. 1985; Kitamoto et al. 1986) and detergent treatment
501 (Glenner et al. 1969; Prusiner et al. 1987).

502 Native PrP protein has been crystallized (Antonyuk et al. 2009) and solved by NMR
503 (Riek et al. 1996; James et al. 1997; Riek et al. 1998; Zahn et al. 2000), but working with
504 non-native and insoluble amyloid forms of proteins is problematic for traditional
505 structural techniques. The secondary conformations found in amyloids were first
506 elucidated in the 1960s and showed a beta-sheet rich structure with the beta-sheet axes
507 perpendicular to the long axis of each fibril (the so-called cross-beta structure) (Eanes &
508 Glenner 1968). Many subsequent studies have borne out the basic conclusion for
509 different animal amyloid and prion proteins (Harper et al. 1997; Sunde et al. 1997;
510 Lyubchenko et al. 2012; Tycko & Wickner 2013; Groveman et al. 2014) with the latter
511 papers clarifying a parallel in-register intermolecular beta-sheet structure for the amyloid
512 forms of these proteins.

513 Amyloid proteins self-assemble into large, complex aggregates and fibrils on the basis of
514 their unusual beta-sheet rich tertiary conformations (Fig. 2). The process of fibril
515 formation has a number of steps (Dobson 2003; Gregersen et al. 2005; Chiti & Dobson
516 2006; Tanaka et al. 2006; Maji et al. 2009; Naeem & Fazili 2011; Eisenberg & Jucker
517 2012; Knowles et al. 2014). One model is presented here, although other models have
518 been proposed (Colby & Prusiner 2011b). In this model, conversion of native to amyloid
519 form is a rare event (Fig. 2A) where the misfolded proteins can associate and cause
520 conformational conversion of other natively-folded proteins (Fig. 2B). Through this
521 process, oligomers are formed (Fig. 2C) that eventually assemble into longer fibrils (Fig.
522 2D). Chaperone proteins and other proteins may be involved in cleaving long fibrils into

523 smaller pieces (Fig. 2D to Fig. 2C). It has been noted that the amyloid oligomer stage
524 (Fig. 2C) is likely the most toxic to cells and tissues (reviewed in Kayed & Lasagna-
525 Reeves 2013 and Verma et al. 2015). It is also worth noting that while amyloid
526 formation is clearly a process that involves cytotoxicity and histotoxicity, production of
527 rod-type and other non-amyloid aggregates is also possible with PrP and disease can still
528 result (Wille et al. 2000).

529 The *Prnp/PRNP* genes in animals and humans encode the PrP protein (Oesch et al. 1985;
530 Basler et al. 1986) and the domain structure of the translated PrP protein (Fig. 3A) has
531 been long studied and dissected for interesting and notable features (reviewed in Colby &
532 Prusiner 2011). The mammalian prion protein, PrP, as shown in Fig. 3A, contains five
533 octarepeats (consensus sequence: PHGGGWGQ) (Brown et al. 1997). The similar length
534 of each repeat and number of repeats found in each protein is suggestive of some
535 important function. The importance of the repeats in PrP is underscored because PrP
536 repeat expansion is associated with dominant inherited prion disease (Wadsworth et al.
537 2003; Prusiner et al. 1998) and removal of the repeats in a mouse model of disease slows
538 progression (Flechsig et al. 2000). The profile of the repeat structures in PrP rose further
539 when it was noted that there are compositional similarities between the repeats in PrP and
540 in the yeast prion Sup35 (Fig. 3B, with similar prevalence to PrP of the amino acids
541 proline, glycine, and glutamine in the repeats, for example, as detailed in the next
542 section). Indeed, in the context of yeast Sup35, its oligopeptide repeat domain (ORD)
543 repeats can even be functionally replaced with PrP repeats and propagation is unimpaired
544 (Parham et al. 2001). And in a result analogous to the *in vivo* repeat expansion
545 experiment, Sup35 aggregates with increasing numbers of PrP repeats have reduced times

546 to fiber formation *in vitro* (Kalastavadi & True 2008). Given the similarity between
547 Sup35 and PrP repeats and the presence of repeat elements in other yeast prion
548 domains—Rnq1 and New1 (Osherovich et al. 2004; Vitrenko et al. 2007)—primary
549 sequence effects could be an important consideration for propagation of prions.
550 However, as discovered in yeast prions (section 4.3 below), primary sequence elements
551 like repeats may instead represent a convenient genetic method of rapidly expanding
552 amino acid compositional biases that lead to prion formation.

553 Other structural features have been noted for PrP as well (Fig. 3A). It is doubly-
554 glycosylated near the cysteines involved in a disulfide bridge and has a GPI-anchor for
555 cell membrane attachment. Unlike the repeat structures noted above, these features have
556 not been generally noted in the yeast prions and so may represent less commonly found
557 domains or characteristics of prion proteins.

558

559 ## 4.3 Structural characterization of yeast prions

560 Although the non-Mendelian cytoplasmic characters [URE3] and [*PSI*$^{+}$] from yeast were
561 shown to be prions in 1994, many aspects of their fundamental biology remained to be
562 worked out. Though Wickner had shown a protein-only inheritance in the yeast prions
563 consistent with that previously proposed in mammalian PrP, whether the yeast prions
564 would share the basic protein structure of an abnormal amyloid fold was not known. The
565 amyloid structure would first be noted for [*PSI*$^{+}$] (King et al. 1997) and [URE3] (Taylor
566 et al. 1999) and the predicted (Ross, Minton, et al. 2005) parallel in-register beta-sheet
567 structure observed for PrP would be noted for [URE3] (Baxa et al. 2007), [*PSI*$^{+}$]

568 (Wickner et al. 2008; Shewmaker et al. 2009; Chen et al. 2009) and others (Chen et al.
569 2009; Engel et al. 2011). Yeast prions, found to generally form amyloid structures, were
570 also protease and detergent resistant (Masison & Wickner 1995).

571 The full history of yeast prion characterization is outside of the scope of this review (for a
572 fuller discussion see Wickner 2012), but I will discuss several key structural and
573 biochemical features of yeast prions beyond amyloid structure in this section.

574 Shortly after Wickner's 1994 paper, it was rapidly noted by Yury Chernoff in Susan
575 Liebman's lab in collaboration with Susan Lindquist's lab, that the chaperone protein
576 Hsp104 was involved in propagating the [*PSI*$^{+}$] prion to daughter cells and cells that mate
577 with [*PSI*$^{+}$] cells (Chernoff et al. 1995; Lindquist et al. 1995) and this process would be
578 mediated by Hsp104's ability to cleave fibrils into smaller pieces (reviewed in Sweeny &
579 Shorter 2016, see also the arrow from Fig. 2D to 2C).

580 The function of yeast prions is a matter of some debate. Unlike the TSEs which greatly
581 hamper neurologic function and are uniformly fatal when symptoms begin, prions in
582 yeast, due to short generation time and rapid growth, could be beneficial (True &
583 Lindquist 2000; Suzuki & Tanaka 2013) or harmful (Nakayashiki et al. 2005;
584 McGlinchey et al. 2011; Wickner et al. 2011). In fact, there is no reason to expect that
585 prions could not be both sometimes beneficial and sometimes harmful to the cell.

586 The normal function of each host protein, Sup35 and Ure2, were exploited as assays for
587 the detection of prion activity as well. Detection of [URE3] relies on growth
588 characteristic of the cells in the presence of a good nitrogen source. [URE3] cells in this
589 circumstance would be able to take up ureidosuccinate, an intermediate compound in

590 uracil biosynthesis, while cells without the [URE3] prion cannot uptake ureidosuccinate
591 (Lacroute 1971). This ability has been used to assay for the presence of the [URE3] prion
592 but it can be a difficult assay to work with (Brachmann et al. 2006). Assaying for [*PSI*$^{+}$]
593 is a much easier-to-interpret test. Because Sup35 is an 'omnipotent suppressor' that can
594 read-through stop codons (Ter-Avanesyan et al. 1994), in a cellular background
595 containing an *ade2-1* (or similar) mutant with a premature stop codon, suppression by the
596 eRF3 function of Sup35 will lead to read-through in prion-containing cells and no read-
597 through in prion-negative cells (Fig. 4A). Because the *ade2* mutant is non-functional
598 without read-through, oxidized P-ribosylaminoimidazole in the adenine biosynthetic
599 pathway will accumulate and the cells will be red in color when plated on limiting
600 adenine (Fig. 4B, right). If the prion state removes active Sup35 from the cell by
601 sequestering it in fibrils, read-through will occur and the cell will remain wild-type in
602 color (Fig. 4B, left).

603 Unusually, both [URE3] and [*PSI*$^{+}$] were found in genetic screens where, uncommonly,
604 a loss of function event for either protein was advantageous to the cell (Lacroute 1971;
605 Cox 1965). In most cases, detecting such a rare loss of function event would be
606 extremely difficult. However, structural studies of [URE3] and [*PSI*$^{+}$] revealed an
607 exploitable feature of these proteins that could help identify other, similar, prions.

608 Sup35, the protein that forms the [*PSI*$^{+}$] prion, features three domains (Fig. 3B): an N-
609 terminal (N) domain that is responsible for prion formation (also called a prion forming
610 domain—PFD—or prion-like domain—PrLD), a charged middle domain (M) and a C-
611 terminal catalytic domain (C) responsible for the nonsense-suppression (eRF3) function
612 of Sup35 (Ter-Avanesyan et al. 1993). The N domain is rich in glutamine and asparagine

613 (Q/N) amino acid residues. Within the N domain, the nucleation domain (ND), the first
614 39 amino acids, is more Q/N-rich than the portion of the N domain immediately after
615 (DePace et al. 1998). This section, the oligopeptide repeat domain (ORD), is also
616 enriched in glutamine and asparagine, but is primarily noted for having a series of 5 ½
617 imperfect repeats (Fig. 3B) (Osherovich et al. 2004; Shkundina et al. 2006). Ure2 also
618 has a substantial Q/N-tract that is required for prion formation (Masison & Wickner
619 1995). What made these Q/N-rich domains of even greater interest was that these
620 domains were modular (the compact Q/N-rich portion of the protein enabled the protein
621 to assume an amyloid shape without contribution from the rest of the three-dimensional
622 structure) and also transferrable (that amyloid/prion forming ability could be fused to
623 many other proteins and cause them to also become amyloid/prion forming) (Li &
624 Lindquist 2000; Baxa et al. 2002). In both the Sup35 and Ure2 yeast prion proteins, the
625 prion domain was also dispensable, and could be deleted without affecting catalytic
626 functions (domains reviewed in Ross et al. 2005).

627 The prion domains of the [URE3] and [*PSI*$^{+}$] prions have a curious conformational
628 property as well. For almost all known proteins, three-dimensional structure and function
629 are inextricably linked to the primary sequence, the ordered series of amino acids. In the
630 beta-sheet rich [URE3] and [*PSI*$^{+}$] prions, it is possible to actually scramble the order of
631 the amino acids in each PFD (using a random number generator) and retain both the
632 amyloid structure and the prion function/effects in the cell (Ross, Edskes, et al. 2005;
633 Ross et al. 2004; Ross, Minton, et al. 2005; Shewmaker et al. 2006).

634 The ability to scramble amino acid order while retaining structure and function is an
635 especially curious property given that, as detailed in section 4.2, Sup35 has been utilized

as a model for examining the role of prion protein repeats in formation and propagation of aggregates (Parham et al. 2001; Dong et al. 2007; Tank et al. 2007; Kalastavadi & True 2008) and the mammalian PrP repeats have been repeatedly suggested to be important for disease (Wadsworth et al. 2003; Prusiner et al. 1998; Flechsig et al. 2000).

In the case of [*PSI*$^{+}$], the two portions of the PFD (the N-terminal ND region and the C-terminal ORD region) have distinct amino acid compositions (Toombs et al. 2011). The distinct compositions seem to relate to different functions of each subdomain: the ND is required for nucleation or formation of the prion and the ORD is required to propagate or maintain the prion (DePace et al. 1998; Osherovich et al. 2004; Shkundina et al. 2006). The ability to scramble prion primary sequence and still generate functional prions led to important experiments, discussed below, useful in understanding yeast prions and in identifying new candidate prions.

4.4 Making predictions: Using biochemical knowledge of known prions to identify other prions and understand the prion structure-function relationship

Given the longer history of study of the animal prions, it might be expected that after Prusiner's prion hypothesis (Prusiner 1982) gained traction, other animal prions would be rapidly discovered. That has not been the case, although some (bottom part of Table 2), including the alpha-synucleinopathies, appear to form *bona fide* infectious prions. Alpha-synuclein, which has no sequence similarity to PrP, has recently been reported using mouse animal and cell culture models of human multiple system atrophy (MSA) as

658 a prion (Watts et al. 2013; Woerman et al. 2015; Prusiner et al. 2015; reviewed in
659 Supattapone 2015). Alpha-synucleinopathies aggregate alpha-synuclein with other
660 proteins in pathological structures called Lewy bodies (Spillantini et al. 1997; Mezey et
661 al. 1998) that are found in Parkinson's disease, MSA, Lewy-body dementia, and some
662 cases of Alzheimer's disease (Yokota et al. 2002). It is likely that other human prion or
663 prion-like diseases may still await discovery. True infectious prions in mammals have
664 not been easily found, but as noted in section 5 below, the enlargement of the prion
665 concept may instead show that other prion-like diseases have been hiding, perhaps, in
666 plain sight.

667 Despite difficulties in identifying new animal prions, a whole host of new candidate and
668 verified yeast prions have been found since Wickner's 1994 recognition of the prion
669 hypothesis in *Saccharomyces*. The ease of genetic screens and manipulation in yeast has
670 made a host of different approaches possible. These studies in turn have led to greater
671 structural insights and each new observation has improved methods for identifying other
672 prions, resulting in more discoveries. The current list of likely yeast prions is ~18 in *S.*
673 *cerevisiae* alone. And because prions are a subset of aggregative proteins that form a
674 major new class of human diseases and the proteins responsible for these human diseases
675 share characteristics with yeast prions, identifying new prions in yeast (reviewed in
676 MacLea & Ross 2011) is a topic of considerable interest with applications in human
677 disease. Several techniques have been used or proposed to identify new prions in yeast:
678 (1) Prion-prion interactions; (2) Q/N-content or other composition; and (3) Other
679 bioinformatics and proteomics methods.
680

4.4.1 Prion-prion interactions help reveal new prions

Prions interact frequently with other prions in yeast, and these interactions can have variable effects on prion formation and propagation (Gonzalez Nelson & Ross 2011). The [*PIN*$^+$]/[*RNQ*$^+$] prion has been most well-studied in its effects on other prions, particularly its ability to promote formation of the [*PSI*$^+$] prion (Derkatch et al. 1997; Derkatch et al. 2000; Derkatch et al. 2001). The identification of [*PIN*$^+$]/[*RNQ*$^+$], described below, allowed Irina Derkatch to perform a genetic screen to identify factors that could substitute for [*PIN*$^+$] in allowing [*PSI*$^+$] formation (Derkatch et al. 2001). This method identified 11 candidate prions, of which one was shown to be prion-like in certain assays but has not been shown to form prions in its native state (New1), and two were identified as likely prions (Swi1 and Cyc8) (Derkatch et al. 2001; Du et al. 2008; Patel et al. 2009). This genetic screen was unique to [*PIN*$^+$] and given that little is known about the seeding or other mechanism responsible for the behavior of [*PIN*$^+$] in the cell, this method has not been used in additional screens.

4.4.2 Q/N or other amino acid composition as a tool for prion identification

[*PSI*$^+$], encoded by the *SUP35* gene in yeast, has a prion-forming domain (PFD) that is both modular and transferable and has an extremely easy-to-use and robust assay for prion formation (Fig. 4 and see above), making it the ideal platform on which to test other candidate prions. A classical experimental scheme using Sup35 in this manner involves replacing the N domain (PFD) of Sup35 (see Fig. 3B) with any candidate ORF and then assessing its function in the *ade2-1* assay conventionally used to monitor [*PSI*$^+$] function

(Fig. 4). Using this scheme, additional prions would soon be identified in yeast, including [*NU*$^{+}$] encoded by New1 (Michelitsch & Weissman 2000) and [*PIN*$^{+}$]/[*RNQ*$^{+}$] encoded by Rnq1 (Santoso et al. 2000; Sondheimer & Lindquist 2000; Derkatch et al. 2001). The PFDs of New1 and Rnq1 were also Q/N-rich and also transferrable, conferring the ability to aggregate even on the green fluorescent protein (GFP) in the absence of Sup35 (Sondheimer & Lindquist 2000; Osherovich & Weissman 2001; Osherovich et al. 2004). The New1 PFD has additional similarities to Sup35, including separation of the formation and propagation functions within the PFD (Osherovich et al. 2004, discussed below for Sup35).

When New1 and Rnq1 were identified and shown to have similar Q/N content and characteristics to Sup35 and Ure2, two large-scale bioinformatics screens looking for Q/N-rich predicted prions in the yeast proteome were undertaken, in Jonathan Weissman's lab (Michelitsch & Weissman 2000) and by Paul Harrison and Mark Gerstein (2003). Melissa Michelitsch found 107 candidate yeast prion proteins, including most (8/11) found by Irina Derkatch, all four of the previously identified prions (Ure2, Sup35, New1, Rnq1) and four that were later shown to be *bona fide* prions (Swi1, Cyc8, Mot3, Sfp1) (Michelitsch & Weissman 2000; Du et al. 2008; Patel et al. 2009; Alberti et al. 2009; Rogoza et al. 2010). Paul Harrison found 172 prion candidates of which 101/172 were found by Michelitsch and 9/11 of the proteins found by Irina Derkatch in her genetic screen (Harrison & Gerstein 2003). All 8 of the proven/likely prions found above were also found in this study (Ure2, Sup35, Rnq1, Swi1, Cyc8, Mot3, Sfp1). Michelitsch and Harrison both identified a large number of candidate prion proteins, but determining which of these candidates to examine further was not obvious given the

methods used. A combination of the bioinformatics screen with an experimental approach was necessary.

The method of fusing prospective candidate PFDs to Sup35 to test prionogenicity and three other aggregation assays were used in a major study out of Susan Lindquist's lab to address this central criticism of previous bioinformatics screens. In this study (Alberti et al. 2009), a computational tool called a hidden Markov model (HMM) was first used to identify the 100 most-similar proteins to Ure2, Sup35, Rnq1, and New1. In a mammoth experiment, each of those 100 ORFs was then tested in four different tests of prion-like activity, and 23 proteins were found that could induce prion formation in the context of Sup35 (Alberti et al. 2009). This method did not identify all potential prions since two known prion proteins, Cyc8 and Mot3, did not show prion activity in this assay. Showing the utility of this combined bioinformatics/empirical approach, although 67/100 of the ORFs had been previously implicated by Michelitsch and Harrison (Michelitsch & Weissman 2000; Harrison & Gerstein 2003), most did not have prion activity in one, two, three, or four of the prion candidate testing methods (Alberti et al. 2009).

The enormous combined screen of Simon Alberti and Randal Halfmann in Susan Lindquist's lab (Alberti et al. 2009) provided a data set of immense value, adding in the experimental results for all four assays of aggregative/prion activity to the computational screens previously conducted. Still, within the data set generated, there was found to be no substantial relationship between the degree of similarity of each of the 100 ORFs to previously known prion sequences with their results in the four assays (Alberti et al. 2009; Toombs et al. 2010; Ross & Toombs 2010). While at first blush this suggests that amino acid composition may not be the main determinant of prion propensity, the

incompleteness of previous knowledge on what made a prion and the small sample size likely meant that the algorithm was not optimized for this situation. What was needed was an experiment that would give scoring values for each amino acid so that an increase or decrease in propensity to form prions could be calculated, without relying on previously discovered yeast prions.

In Eric Ross's laboratory, Trey Toombs used a scrambled version of Sup35 and replaced two short segments with a random sequence to generate two libraries of mutants (Toombs et al. 2010; Ross & Toombs 2010). For each library, different regions of the Sup35 protein nucleation domain were modified and he then compared (in each library) the amino acid composition for a naïve subset of clones (with no selection) with a subset that could form prions and generated a prion-propensity score for each amino acid. This allowed regions and whole ORFs and proteomes to be scanned and scored to evaluate overall predicted prion propensities. Using another algorithm, FoldIndex, that measures order/disorder propensity (Prilusky et al. 2005), Toombs found that known yeast PFDs had extended disordered regions with only modest prion propensities (Toombs et al. 2010; Ross & Toombs 2010). Although not a perfect predictor, this method did improve (Toombs et al. 2010) on the blind HMM method used in Lindquist's lab and was reasonably effective at predicting prion propensities for the proteins examined in the four assays of aggregative/prion function (Alberti et al. 2009). The resulting algorithm for screening yeast proteins for prion propensity was named PAPA (Toombs et al. 2010; Ross & Toombs 2010; Ross et al. 2013).

The Toombs experiment measured, by its design, the combined processes of prion formation and prion propagation or maintenance. A follow-up study showed that the two

774 subdomains within the PFD of Sup35 had amino acid compositions that were not
775 identical. That is, the composition of the ND (nucleation domain responsible for
776 formation) and the ORD (responsible for maintenance) of Sup35 were different, and
777 therefore propagation of prions to daughter cells had slightly different compositional
778 requirements than nucleation (Toombs et al. 2011). Further work addressed this
779 compositional bias and allowed calculation of separate prion maintenance propensities
780 (MacLea et al. 2015), which may in the future allow these processes to be better dissected
781 and lead to more accurate prediction algorithms for fully-functional prions.

782

783 **4.4.3 Other bioinformatics and proteomics methods for prion identification**
784

785 Numerous algorithms have been developed to predict protein aggregation propensity,
786 chiefly using the mammalian amyloids as a basis. Algorithms including TANGO
787 (Fernandez-Escamilla et al. 2004), Zyggregator (Tartaglia et al. 2008), BETASCAN
788 (Bryan et al. 2009), Waltz (Maurer-Stroh et al. 2010) and ZipperDB (Goldschmidt et al.
789 2010) have been somewhat successful at finding known amyloids in mammalian
790 databases, but have had less utility in identifying yeast prions. Although there is
791 probably more to the story, the amyloidogenesis in both systems is thought to be rather
792 different. Mammalian amyloids appear to require a shorter, highly amyloidogenic
793 stretch, while yeast prions appear to require longer stretches of modest prion propensity
794 with intrinsic disorder as estimated by FoldIndex (Esteras-Chopo et al. 2005; Prilusky et
795 al. 2005; Ross & Toombs 2010). Newer algorithms focused on yeast prions, such as
796 ArchCandy, which incorporates three-dimensional modeling, may prove useful as well

(Bondarev et al. 2013) but at the moment no verified new prions have been identified using these methods.

Simulations of molecular dynamics for short peptide stretches found commonly in mammalian prions were used in the creation of some of the algorithms above and have shed some light on how the conformational conversion process from native to amyloid shape may occur at the molecular level. Similar simulations for the Q/N-rich prions have also been undertaken (Halfmann et al. 2011; Berryman et al. 2011). Proteomics methods including two-dimensional gels and mass spectrometry have been proposed and used in small studies, but the insolubility of the amyloidogenic proteins makes these kinds of techniques very tricky to interpret. Other methods may prove useful in the future for identification of more amyloid and prion proteins. Any such method developed will need to work around difficult intrinsic properties of these proteins, including insolubility, protease and detergent resistance, and more. Methods that are not biased in the same ways as earlier studies (looking only at Q/N-rich proteins, relying on fusion to Sup35 for an assay, etc.) will likely yield the most fruit in years to come. One such study that exploits the difficult intrinsic properties of prion and amyloid proteins was recently published (Kryndushkin et al. 2013) and may be a useful template for future proteomics experiments to identify new prions or similar proteins.

4.5 Strains

In the previous parts of section 4, overall physical structures of animal (4.2) and yeast (4.3) prions have been examined, showing key features of these proteins, *e.g.,* amyloid

820 structure, staining properties, protease and detergent resistance, domain structures, repeat
821 sequences, and amino acid compositions. These properties of 'what makes a prion' were
822 the initial seeds upon which further studies have been built. In learning to identify new
823 prions, chiefly in yeast (4.4), new features of both yeast and animal prions and amyloids
824 have been noted, further expanding the field's knowledge of the essential characteristics
825 and diversity of prions and amyloids. One key, but unusual, feature of prions has not yet
826 been discussed: distinct prion strains.

827 Like other pathogens, prions have strain differences and these strain differences are
828 propagated when the prions are transmitted. This was first noted in scrapie (Dickinson &
829 Meikle 1969; Fraser & Dickinson 1973). Animal prion strains appear to be caused by
830 conformational diversity (different stable forms with tertiary conformational variability)
831 being inherited more or less faithfully (Bessen & Marsh 1994; Telling et al. 1994;
832 Collinge et al. 1996; Peretz et al. 2001; Colby & Prusiner 2011a). Yeast prions have
833 widely appreciated strain differences as well (King & Diaz-Avalos 2004; Tanaka et al.
834 2004; Tanaka et al. 2006; Marcelino-Cruz et al. 2011; Huang et al. 2013) that appear to
835 be passed vertically and can be passed *ex vivo* cell to cell using traditional experimental
836 techniques as well. Because prions are not easily passed horizontally in yeast it is unclear
837 whether strains can be naturally transmitted this way.

838

839

5. The Enlarging Prion Concept in Disease and Beyond

5.1 Introduction

Prion diseases such as the TSEs were ultimately identified and set apart from other diseases on the basis of their etiology by a 'proteinaceous infectious particle' or prion. While this was a useful designation in the early years of prion studies, when scientific consensus on the existence of prions was far from sure, it is now becoming clear that the segregation of prions from other agents of pathological protein aggregation is inappropriate. For example, non-infective amyloids such as amyloid precursor protein (APP) and tau, when injected directly into the central nervous system of other animals, appear to be able to cause disease (Haass et al. 1995; Clavaguera et al. 2009). Human patients have also acquired Lewy-body type pathologic inclusions from brain grafts (Kordower et al. 2008). From these and other observations (*e.g.,* Jucker & Walker 2011; Eisenberg & Jucker 2012), it appears clear that the line separating the infectious prions from the non-infectious amyloids or pathologic aggregates is thinner than previously thought. As a result, the consensus is that the prion concept itself is enlarging to encompass other diseases of aberrant protein aggregation as well (Colby & Prusiner 2011b; Walker & Jucker 2015).

5.2 Developing a definition of a general category of prion-like conformational states

It was recently proposed that a new category of prion and prion-like diseases should together share certain essential characteristics (Colby & Prusiner 2011b). (1) A post-translational conformational change occurs in a native protein to a form with high beta-

sheet content; (2) Oligomers are formed from the high beta-sheet protein forms and are toxic to cells; (3) Polymerization into fibrils results in reduced toxicity of the high beta-sheet forms; (4) 'Plaques,' 'tangles,' or 'bodies' result from sequestration of the fibrils inside and outside of cells, in the central nervous system; and (5) Mutations in these proteins may cause familial heritability of these traits.

5.3 Prion-like proteins, quasi-prions, and prionoids

A growing awareness of the broad swath of prion-like phenomena has necessitated some new terms to distinguish these categories. Paul Harrison's lab has suggested the categories of prion and prion-like proteins, with the latter category made up of quasi-prions and prionoids (Harbi & Harrison 2014). Briefly, prions have firm evidence of prion behavior, with fully infective particles made *in vitro* (strongest evidence, *e.g.,* Sup35) or not (weaker, *e.g.,* Cyc8). Quasi-prions behave similarly to prions but do not meet the infection requirements of a prion, but can still pass the quasi-prion to progeny (for example, the likely prionogenic proteins from the Alberti *et al.* 2009 study or RepA-WH1 in bacteria). Prionoids have been shown to propagate between cells in multicellular organisms (for example, Tau in Alzheimer's disease). Regardless of the specific nomenclature, the rising realization in the aggregation and prion communities that there is overlap and crosstalk between the fields that may allow leaps in one area to rapidly cross-pollinate to another area across these categories make an understanding of the relatedness of the concepts especially apt and timely. For example, in the next section, the application of discoveries in the yeast realm to studies of familial human diseases illustrate that these prion-like phenomena clearly share a biochemical and cellular basis.

5.4 The intersection of animals and yeast: Studies of yeast prions have lead to understanding of human amyloid diseases

Yeast prions have helped us to find amyloid proteins in humans. Although PrP is by far the most well-studied human prion protein, Q/N-rich proteins are overrepresented in the human proteome (Michelitsch & Weissman 2000; Harrison & Gerstein 2003) and study of these proteins in the context of yeast has been useful for identifying aggregating proteins in humans (reviewed in Cascarina & Ross 2014). All of the following suspect amyloid proteins were tested in the yeast prion model. For example, amyloidogenic proteins generated from mutant TDP-43 alleles were linked with amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), Alzheimer's and Parkinson's diseases (Neumann et al. 2006; Lagier-Tourenne et al. 2010; Johnson et al. 2009; Da Cruz & Cleveland 2011; Johnson et al. 2008). Mutations in FUS/TLS, EWSR1, and hnRNPA1 and hnRNPA2B1 were shown to cause ALS in some families (Sun et al. 2011; Kwiatkowski et al. 2009; Vance et al. 2009; Daigle et al. 2013; Couthouis et al. 2012; Kim et al. 2013). Additional human amyloid proteins have been found in this way as well (reviewed in Cascarina & Ross 2014), and it is extremely likely that additional discoveries will be made in the coming years by fusing advanced genetic and pedigree analysis of humans with the experimental virtues of the simple, well-worn yeast prion analysis system. In undertaking studies such as these, it is interesting to note that these human proteins, in large part, share more sequence/structure characteristics with the yeast prions than they do with PrP, demonstrating that fundamental biology is at work, probably for all eukaryotic cells and perhaps for all cells.

5.5 What ties together prion-like phenomena

Abnormal accumulation of disease-specific protein aggregates is a hallmark of most neurodegenerative disorders. These include Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple system atrophy (MSA), frontotemporal lobar degeneration (FTLD), and others. The proteins implicated in these disorders are numerous (reviewed in Walker & Jucker 2015) but they all involve aggregation-prone proteins, many with prion-like domains, ability to form beta-sheet rich secondary conformations, and the ability to spread locally within brain regions and form plaques or similar deposits with concomitant toxicities. In short, they meet the requirements set above for prion-like behavior (section 5.2) (Colby & Prusiner 2011b). What all of these disease-causing proteins fundamentally share is that they are based on seeded aggregation of proteins. As the field moves forward, grouping the diseases together that are caused by seeded abnormal protein aggregation is perhaps the best starting place for a new understanding of the prion concept. What Walker and Jucker have referred to as a 'proteinaceous nucleating particle' (Walker & Jucker 2015) brings the prion diseases and the non-prion amyloid diseases together with yet-to-be-discovered variants under the umbrella term 'prion.' While this term has not yet been widely used to encompass infectious and non-infectious aggregating proteins (and indeed whether the term is ever used in that fashion), the enlargement of the prion concept and the acknowledgement that there is relatively little difference between prions and non-infectious amyloids has already begun.

6. Concluding Remarks

In this review, I have discussed the history of the discovery of prions in mammals and the resulting recognition that previously discovered but unexplained non-Mendelian traits in the baker's yeast *Saccharomyces cerevisiae* represented prions as well. The essential genetic, biochemical, and biophysical features of the mammalian prions and amyloids, and the yeast prions and prion-like molecules, while broadly similar, show significant differences as well. Despite this, understanding of the simple yeast prion system has allowed for major health and basic science discoveries in the mammalian context and insights from mammals have informed the studies of prion proteins in yeast. The collective discoveries in this area have grown larger through a recognition that aggregative proteins form a larger constellation of related phenomena (including many diseases). Because of this, the scientists and physicians studying aggregating proteins responsible for human and animal disease, whether infective or not, would do well to familiarize themselves with the literature across the whole gamut of prion, prion-like, and amyloid proteins, because these phenomena clearly demonstrate fundamental similarity at the cellular level that can be exploited to solve problems in all parts of the field.

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1933 **Table 1.** Prevailing notions of natural causes of disease with notable milestones.

Time frame	Agent	Advocate(s)	Physical Basis
Ancient until 19th century	Miasma	Galen of Pergamon, Indian and Chinese philosophers	Bad airs
Ancient until 19th century	Contagion	Fracastoro and others	Direct contact with sick people
1836	Living germ or seed	Bassi	Fungal pathogen, no microscopic evidence
1865-1870	Microbe	Pasteur	Fungal pathogen
1876	Bacterium	Koch	Anthrax bacillus
1898	Virus	Beijerinck, Loeffler and Frosch	Tobacco mosaic virus (TMV), Aphthovirus
1942	Virus	Cohen and Stanley	TMV composed of nucleic acid and protein
20th century	Slow virus	Many	Virus composed of nucleic acid and protein with long incubation period
1982	Prion	Prusiner	Animal disease caused by protein only (no nucleic acid)
1994	Prion	Wickner	Yeast infectious protein (no nucleic acid) explains unusual genetics of [*PSI*$^{+}$], [URE3] traits

1934

1935 **Table 2.** Prion diseases in non-human mammals and humans (After Colby & Prusiner
1936 2011).
1937

Animal Disease	Mechanism	Animal(s)
Scrapie	Somatic mutation in *Prnp* gene or spontaneous conversion of normal PrP^{C} to abnormal PrP^{Sc} or infection from other infected animals	Sheep, goats
Bovine spongiform encephalopathy (BSE)	Infection or sporadic	Cattle
Transmissible mink encephalopathy (TME)	Infection from sheep or cattle	Mink
Chronic wasting disease (CWD)	Infection or possibly sporadic	Cervids (deer, elk)
Exotic ungulate encephalopathy	Infection with prion-contaminated meat and bone meal (MBM)	Ungulates (oryx, nyala, greater kudu, etc.)
Feline spongiform encephalopathy (FSE)	Infection with prion-contaminated meat or MBM	Domestic cats, various wild cats
Proposed canine spongiform encephalopathy	Unknown, based on a single case report	Domestic dogs
Human Disease	**Mechanism**	**Specific Hosts**
Kuru (extinct?)	Ritual funerary cannibalism	Fore tribe, Papua New Guinea
Sporadic Creutzfeldt-Jakob Disease (sCJD)	Somatic mutation in *PNRP* gene or spontaneous conversion of normal PrP^{C} to abnormal PrP^{Sc}	All humans

Familial CJD	Germline mutation in *PNRP* gene	Humans from CJD families
Variant CJD (vCJD)	Infection from consumption of meat from BSE cattle	All humans
Iatrogenic CJD (iCJD)	Infection from contaminated medicines or medical equipment	All humans
GSS	Germline mutation in *PNRP* gene	Humans from GSS families
Fatal Familial Insomnia (FFI)	Germline mutation in *PNRP* gene	Humans from FFI families
Sporadic fatal insomnia (sFI)	Somatic mutation in *PNRP* gene or spontaneous conversion of normal PrP^{C} to abnormal PrP^{Sc}	All humans
Multiple system atrophy	Mutant alpha-synuclein infection in mice/cultured cells (artificial model) (reviewed in Supattapone 2015)	Unknown
Other diseases	Growing recognition of prion-like and amyloid proteins in disease and other pathological changes in protein conformation	Unknown

1938

Figure Legends

Figure 1. Brain effects of CJD, a transmissible spongiform encephalopathy, in humans. **(A)** Diffusion-weighted magnetic resonance (MRI) image of a patient who presented with a rapidly-progressive dementia, with initial hallucinations and behavioral change that progressed to a mute, akinetic state with myoclonus. Right cortical and striatal high signal is consistent with a diagnosis of sporadic-type Creutzfeldt-Jakob disease (sCJD). Photo courtesy of Dr. Laughlin Dawes and Wikimedia user Filip em, 2008. **(B)** Hematoxylin-eosin stained cortex of patient with variant Creutzfeldt-Jakob (vCJD) disease with florid plaques. Photo is in the public domain.

Figure 2. Process of assembly of toxic oligomers, protofilaments, and fibrils in amyloid-based diseases, including prion diseases. **(A)** Spontaneous conversion between a native or normally-folded protein state into an abnormal or amyloid state (beta-sheet rich) are very rare. Both forms are stable states. **(B)** Once an abnormal amyloid form of a protein is present in a cell, when it encounters a natively-folded protein it is capable of causing a conformational change in which the native protein assumes an amyloid structure. **(C)** When amyloid-structured proteins encounter each other, they have a tendency to aggregate and form, initially, short stretches of dimers, trimers, and oligomers. Evidence suggests these oligomers are more toxic to the cell than monomers or larger filaments (*e.g.,* Simoneau et al. 2007; reviewed in, *e.g.,* Verma et al.). **(D)** Oligomers that pick up additional monomers or oligomers may assemble into larger protofilaments and then fibrils that can be extremely large. These fibrils are often hallmarks of amyloidoses and can be visualized in histopathologic sections with various straining and imaging techniques. Chaperones (such as Hsp104 in yeast) are capable of cleaving larger fibrils

into shorter pieces, which appears to be required for proper maintenance of the prion during cell division.

Figure 3. Domain structures of canonical mammalian and fungal prions. Repeat domains are noted with single-letter amino acid abbreviations for repeat structures in the protein sequences. **(A)** Human Prion Protein (PrP), which can interconvert between normal PrP^{C} and abnormal PrP^{Sc} protein variants. Abbreviations: SP, signal peptide; S-S, disulfide bridge; GPI, Glycophosphatidylinositol anchor. **(B)** Yeast prion protein Sup35 (eRF3) which can give rise to the [*PSI*$^{+}$] prion. Abbreviations: N-domain, prion domain; ND, nucleation domain region of the N-domain; ORD, oligopeptide repeat domain region of the N-domain; M domain, middle domain; C domain, catalytic domain.

Figure 4. Assay for presence of the yeast [*PSI*$^{+}$] prion using the *ade2-1* mutant nonsense suppression (eRF3) function of Sup35. **(A)** Schematic diagram for *ade2-1* generation of color phenotypes in the presence or absence of the [*PSI*$^{+}$] prion. **(B)** Examples of red/white color selection using the *ade2-1* assay. Left, mutant forms of Sup35 that are [*PSI*$^{+}$] in this assay are compared with the control wild-type [*PSI*$^{+}$] prion, plus or minus curing with guanidine hydrochloride (GdHCl). Right, mutant forms of Sup35 that are [*psi*$^{-}$] (non-prion) are shown.

Figure 1.

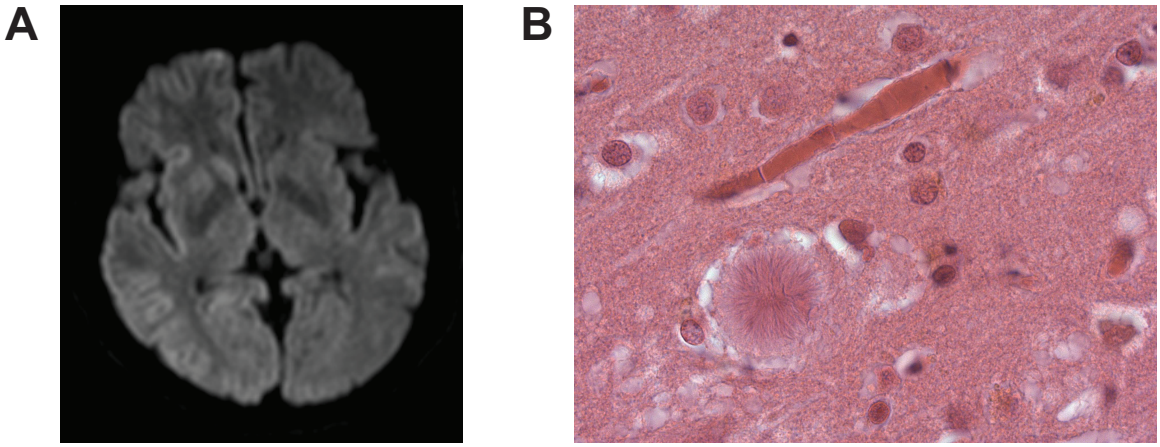


Figure 2.

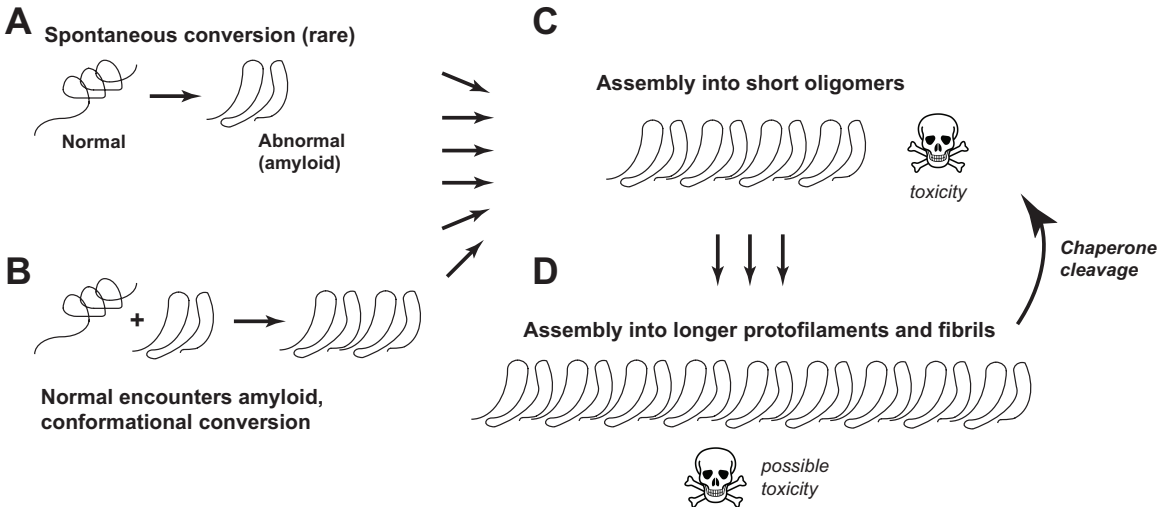
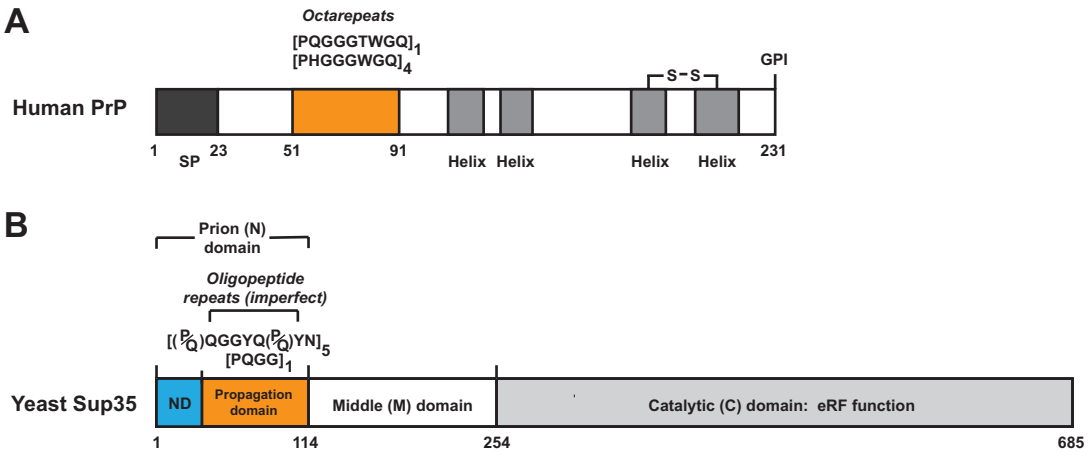


Figure 3.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[PHGGGWGQ]4
GPI
S–S
Human PrP
1
SP
23
51
91
Helix
Helix
Helix
Helix
231
B
Prion (N) domain
Oligopeptide repeats (imperfect)
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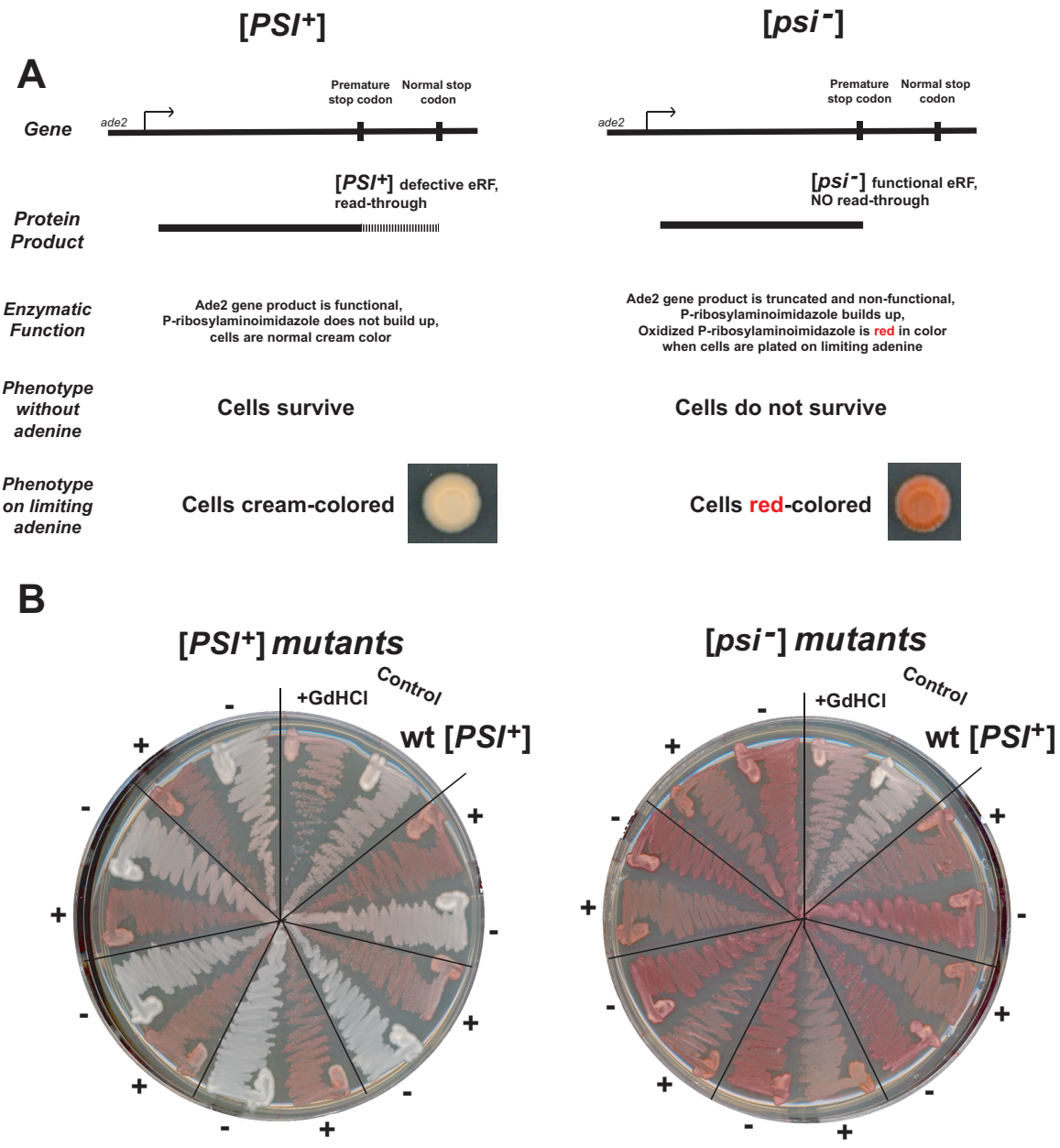
[PQGG]1
Yeast Sup35
ND
Propagation domain
Middle (M) domain
Catalytic (C) domain: eRF function
1
114
254
685

1996 **Figure 4.**



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