DISSERTATION

YEAST PRION PHYSIOLOGY

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2016

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ABSTRACT

YEAST PRION PHYSIOLOGY

Prions, or proteinaceous infections, are caused by proteins that have the unique ability to adopt an alternative, self-replicating structure. These self-replicating structures are the causative agent of a number of mammalian diseases including Bovine spongiform encephalopathy, Creutzfeldt-Jakob disease, and Kuru. More recently, yeast were discovered to carry at least a dozen proteins capable of making this structural conversion. Yeast prions are unique in that their prion-forming domains are intrinsically disordered domains, with unusual compositional biases.

This thesis addresses two broad questions about yeast prion physiology. First, a recent mutagenic screen suggested that both aromatic and non-aromatic hydrophobic residues strongly promote prion formation. However, while aromatic residues are common in yeast prion domains, non-aromatic hydrophobics are strongly under-represented.

The second chapter of this dissertation explores the effects of hydrophobic and aromatic residues on prion formation. Insertion of even a small number of hydrophobic residues is found to strongly increase prion formation. These data, combined with bioinformatics analysis of glutamine/asparagine-rich domains, suggest a limit on the number of strongly prion-promoting residues tolerated in glutamine/asparagine-rich domains. Recent studies have demonstrated that aromatic residues play a key role in the maintenance of yeast prions during cell division. Taken together, these results imply that non-aromatic hydrophobic residues are excluded from prion

domains not because they inhibit prion formation, but instead because they too strongly promote aggregation, without promoting prion propagation.

Despite more than 20 years of research, we still don't know why yeast carry so many prion and prion-like domains. It has been proposed that prions may serve some biological function. Chapter Three presents progress on two lines of investigation designed to resolve this issue First, a novel bioinformatics algorithm (GARRF) is used to screen a wide range of proteomes to find examples of Q/N rich domains outside of *Saccharomyces cerevisiae*. Identifying other species that carry these unusual regions provides insight into their role in cellular biology. We find a wide range species carry prion-like domains at levels comparable to *Saccharomyces cerevisiae*, and a small number carry up to an order of magnitude more.

Second, currently researchers rely primarily on yeast genetic methods to discover and monitor prions. These methods have a number of drawbacks, including a glacially slow readout time. Chapter Three reports on progress towards the development of a novel fluorescence based prion assay. This assay takes advantage of bi-molecular fluorescence complementation, a technique that uses complementary fragments of a fluorescent protein to indicate when two interacting domains are in proximity to one another. When completed, this assay will provide a means to monitor protein aggregations that is both faster and more sensitive than any existing assay.

ACKNOWLEDGMENTS

I would like to thank my mentor, Eric Ross, for what has been one of the defining experiences of my life. I use the skills and lessons learned in your lab every day in my professional life. You changed the way I think about basically everything. I can never thank you enough.

I would also like to thank my committee members, Dr. Robert Woody, Dr. Olve Peersen, and Dr. Mark Zabel, for their guidance, advice and also their abundant patience.

I would like to thank my family, who have been politely nodding while I ramble on about yeast prions for years now. In particular, I need to thank my parents, Rickardo and Marilyn Gonzalez Nelson, who never stopped believing I would finish this thing, all evidence to the contrary. I could never have finished this without the help of Holly Panetta, who inspired me to go back to school, and provided the support that allowed me to finish. My daughters, Ruby and Lucia, who put up with a crabby, stressed, distracted dad for many years. On the upside, you get a copy of this awesome book.

I would finally like to thank all of the members of the Ross lab who provided me with helpful input, discussions, and comic relief.

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CHAPTER 1: INTRODUCTION

Mammalian Prions

Scrapie is a fatal neurodegenerative disease known to infect sheep herds for at least two centuries [6]. Although symptoms vary, the disease generally causes head and neck tremors, itching, weight loss, lip smacking and other unusual behaviors. The term scrapie derives from the sheep's tendency to compulsively scrape their skin and wool off on fence posts. Scrapie was not intensively studied until the middle of the twentieth century, when it was noticed that its brain histology was remarkably similar to the newly discovered human neurodegenerative disease, kuru [7, 8]. Kuru is a progressive, fatal, neurodegenerative disorder found only among the Fore population of New Guinea. Both diseases cause neuron cell death, resulting in a spongy pattern in brain tissue (see Figure 1.1). Both diseases also appeared to have unusually long incubation periods, sometimes requiring years between infection and disease onset. Initially, the causative agent behind these diseases was assumed to be some kind of slow virus [8-10]. However, further study revealed that the causative agent of scrapie had a number of unusual properties, not all of which were consistent with a virus. Brain extracts from carriers retained their infectivity after prolonged formaldehyde treatment (35% formaldehyde treatment over 3 months) [11],

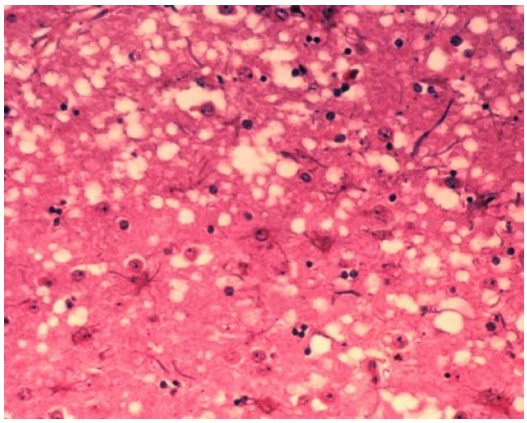

Figure 1.1 A micrograph of brain tissue from a cow infected with bovine spongiform encephalopathy. The white spots are actually microscopic holes that give the brain a spongy appearance. Reproduced from the Public Health Image Library, APHIS: http://www.aphis.usda.gov/lpa/issues/bse/bse_photogallery.html

treatment with UV light [12] and heat [6]. The causative agent was also unusually small for a virus, and resistant to nucleases [12, 13]. Taken together, these studies implied that the causative agent does not contain a nucleic acid component, like other infectious diseases. However, scrapie transmits in series, indicating self-replication, so it was unlikely that the causative agent was a toxin or an environmental factor. In 1967, Professor Griffith, a mathematician at Bedford college, London, proposed several mechanisms that could explain scrapie's odd behavior without overturning the basic tenants of molecular biology [14]. One of these mechanisms has come to be known as the "protein-only hypothesis", the theory that scrapie is caused by a misfolded protein, and that this misfolded protein can catalytically convert non-infected proteins, thus replicating itself (see Figure 1.2).

Although it was initially controversial, two decades worth of supporting evidence, combined with an inability to isolate a virus that causes scrapie, has largely vindicated the protein-only hypothesis. The protein responsible for scrapie, dubbed PrP (for prion protein) was first isolated and identified in 1982 by the Prusiner lab [15, 16]. The gene encoding PrP was identified in 1985 [17, 18]. This gene was subsequently demonstrated to be necessary for disease transmission using transgenic mice lacking the PrP gene [19]. PrP is a mostly alpha-helical, 209-residue protein with a molecular mass of 35-36 kDa, one disulfide bond, two N-linked glycosylation sites, and a glycophosphatidylinositol anchor. In its infective state PrP aggregates into rod-like fibers approximately 25 nm in diameter with highly variable lengths (50-275 nm) [20]

Protein conversion from the native to infectious form was first recapitulated in vitro by Kocisko et al. [21]. Using cell lysate from healthy brains as substrate, they generated infectious material using PrP^{sc} as seed material in a stoichiometrically dependent manner. The process of in

vitro PrP^{sc} generation was further refined by the finding that repetitive cycles of growth and sonication exponentially amplified the amount of infectious material generated, presumably by severing prion fibers into multiple seeds that template further growth [22].

In recent studies wild-type mice were infected with PrP^{sc} that was generated in vivo, both from mammalian-derived protein [23, 24], and recombinant protein [25]. In these studies, efficient infection required co-inoculation with polyanionic molecules like mRNA. The exact role these co-factors play is not well understood. Most likely they act as catalysts or as scaffolds for the recruitment of PrP^{c} to PrP^{sc}.

Subsequent work has shown that all of the TSEs are caused by PrP. This includes the four human prion diseases kuru, Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia, as well as bovine spongiform encephalopathy (affecting cows), chronic wasting disease (affecting deer and elk), scrapie (affecting sheep), transmissible mink encephalopathy (affecting mink) and feline spongiform encephalopathy (affecting several cat species). To date there is no treatment for any of these invariably fatal diseases.

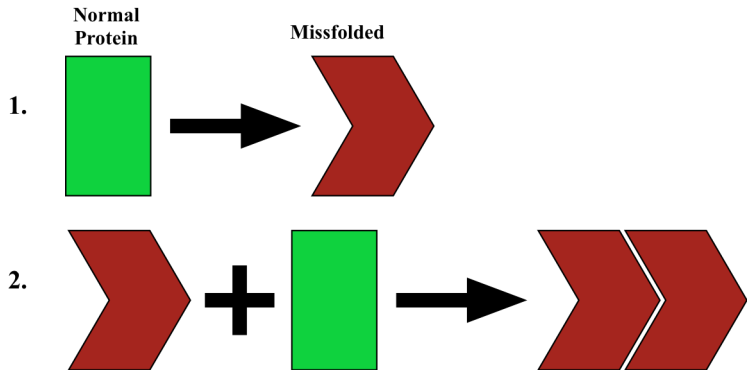

Figure 1.2 An diagram illustrating the protein only hypothesis. Griffiths hypothesized that scrapie may be caused by a self-replicating protein structure. Prions were hypothesized to function in two steps. 1. A protein spontaneously adopts an alternate structure. 2. The misfolded protein converts another protein converting to the alternate structure.

It should be noted that although the protein-only hypothesis is largely accepted as dogma, there are still holdouts. The Manuelidis laboratory, at Yale Medical School, continues to publish alternative putative causative agents, including a 25-nm virion [26] and nuclease-resistant circular DNA fragments [27].

PrP Structure

The structure of the native form of PrP is well understood [28, 29]. It can be subdivided into an intrinsically disordered N-terminal region (residues 23-125) and a globular domain consisting of three α-helices (approximately amino acids 144-154, 175-193 and 200-219) and an antiparallel β-sheet (amino acids 128-131 and 161-164). A disulphide bridge links the second and third helix.

Despite several decades of structural research, we still lack a high-resolution structure of the infectious form of PrP. PrP^{sc} adopts an amyloid-like structure that presents four major obstacles to elucidating its structure. First, PrP^{sc} forms large aggregates, some greater than 400 kD, which are challenging for many conventional biochemical techniques. Second, PrP^{sc} aggregates are hydrophobic, precipitating out of solution and resisting re-suspension in a number of detergents, making many spectroscopic techniques difficult or impossible. Third, PrP^{sc} aggregates are structurally heterogeneous. At the molecular level, individual PrP peptides carry varying degrees of glycosylation and structurally, aggregates vary significantly in size. Finally, PrP^{sc} does not just adopt a single alternative structure. Rather, PrP^{sc} is capable of adopting multiple self-propagating structures referred to as strains. In vivo, strains manifest distinct incubation periods, attack rates, and patterns of neuronal damage. In vitro, H/D exchange experiments have shown that these strains are structurally distinct [30]. Making matters more

complicated, there is evidence that even within a given strain, prion fibers are not perfectly isomorphic, but rather each strain is an ensemble of structures competing to recruit monomeric PrP (for a good review see Collinge et al.) [31].

Despite these difficulties, researchers have pieced together a general understanding of the structure of PrP^{sc} using low-resolution techniques. In proteolytic studies, PrP^{sc} has proven generally resistant to all forms of proteolysis [32]. However, prolonged proteinase K treatment results in a truncated form of the protein, referred to as PrP27-30, that retains its infectivity [33]. This region spans approximately residues 90-209. Truncation experiments confirm this region as the minimum required to propagate PrP^{sc} in a cell-free assay [34, 35]. Antibody mapping confirms that residues 90-120 form an inaccessible core of the PrP^{sc} fiber, with most of the carboxy-terminus of PrP^{sc} inaccessible to antibody binding as well [36]. Spectroscopic techniques including circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR) reveal that PrP^{sc} is rich with beta sheets, approximately 43-47%, with significant amounts of turns and random coil (31-57%), and little or no alpha helix (0-17%) [37, 38]. X-ray fiber diffraction confirms these findings with reflections indicative of a cross-beta sheet [39, 40].

Electron crystallography performed on thin sheets of 2D PrP^{sc} crystals provided the basis for a high-resolution model of PrP 27-30 [41]. Researchers proposed a left-handed β-helix clustered into trimeric columns. In this model, the carboxy-terminus of the protein retains most of its native structure with minimal rearrangement. The fiber grows by stacking β- helix trimers on top of one another.

In a separate study, Surewicz et al. used site-directed spin labeling and EPR spectroscoscopy to probe the molecular structure of amyloid generated from recombinant PrP [42]. Their data suggest a parallel in-register β-sheet that runs the length of the PrP protein. This

model necessitates a major refolding of the C-terminal domain during the transition from PrP^{c} to PrP^{sc}. This data agrees with data generated from a number of other prion-forming proteins that have also been shown to adopt parallel in-register beta sheets [35, 43-46].

It is difficult to compare these models as they are both based on low-resolution techniques and have incongruities with existing structural data. Specifically, the β-helix model proposed by Govaerts et al. leaves the C-terminal region of PrP fairly solvent exposed, a finding that is contradicted by proteolytic data showing the C-terminal region to be solvent protected. Conversely, the in-register β-sheet model is comprised almost entirely of β-sheet. This contradicts spectroscopic methods like circular dichroism and FTIR, both of which show significant amounts of α-helix content in prion fibers [37, 38].

The Discovery of Yeast Prions

The first two *Saccharomyces cerevisiae* prions, [PSI^{+}] and [URE3], were initially identified in screens for yeast phenotypes unrelated to prion formation [47, 48].

The [PSI^{+}] phenotype was discovered in a screen for nonsense suppressors; it was notable for the fact that the phenotype was caused by a dominant non-Mendelian cytoplasmic genetic element [47]. The [PSI^{+}] phenotype is caused by the aggregation of the Sup35p protein (Table 1.1), a subunit of the eRF3 translation termination factor. [PSI^{+}] cells experience intermittent, genome-wide stop-codon readthrough, resulting in the translation of cryptic genetic information.

The [URE3] phenotype was identified in a somewhat less direct manner. Ura2p (not to be confused with Ure2p) is a protein that acts as an aspartate transcarbamoylase (ATCase), producing ureido succinate from carbamyl phosphate and aspartate, a necessary step in pyrimidine biosynthesis [49]. ura2Δ mutants will grow in the presence of ureido succinate,

however, ureido succinate uptake is disrupted by the presence of good nitrogen sources, like ammonia. In 1971 F. Lacroute et al. isolated ura2Δ strains that grew regardless of nitrogen source. In some of these strains, designated [URE3], the phenotype was caused by what appeared to be a dominant non-Mendelian genetic element [48]. [URE3] is the result of the aggregation of the Ure2p protein (Table 1.1). Ure2p serves several functions, including acting as a negative regulator of nitrogen catabolism, preventing the uptake and catabolic metabolism of poor nitrogen sources (i.e. ureido succinate) when good sources of nitrogen are unavailable. When Ure2p is sequestered into prion fibers, the cell takes up ureido succinate regardless of the available nitrogen sources.

In 1994 the Wickner lab demonstrated that the [URE3] phenotype has three traits that have subsequently become the identifying hallmarks of yeast prions [50]. First, the frequency of the [URE3] phenotype increased in populations where Ure2 was overexpressed, presumably because increasing the concentration of Ure2p increases the likelihood of prion nucleation. Second, the Ure2 gene is required for the propagation of the [URE3] prion, and the [URE3] phenotype is identical to the loss-of-function mutation of Ure2p, as you would expect if the protein were being sequestered in amyloid-like aggregates. Finally, [URE3] spontaneously occur in approximately 1 in 10^6 yeast cells. These prions can be cured by treatment with low concentrations of GuHCl. If yeast are returned to normal conditions

Table 1.1 Examples of the yeast prion naming scheme. Note that the protein of a gene is followed with a lowercase p. Prions are epigenetic elements, denoted with brackets.

Gene Name	Protein Name	Prion Name
URE2	Ure2p	[URE3]
SUP35	Sup35p	[Sup35$^+$]

after curing, approximately 1 out of 10^6 cells will spontaneously revert to the prion state. If the phenotype were caused by a nucleic acid-based infection, re-infection would require a second round of inoculation. Because prions are caused spontaneously by native proteins, infection occurs at a low rate, even when yeast are not transfected with infectious material.

Major Advances in Yeast Prion Biology

Saccharomyces cerevisiae provides a uniquely powerful tool in the study of cellular physiology and its genetic underpinnings. As eukaryotes, they share many of the same fundamental biological processes as mammalian cells. However, they are also single-cell organisms that are readily cultured, reproduce quickly and are amenable to genetic manipulation. Not surprisingly, in the past two decades, a number of major advances were made in the yeast prion systems. In fact, the protein-only hypothesis was initially proven in a yeast system: The process of amyloid formation was first recapitulated in vitro using recombinant Sup35p [51] and shortly later with Ure2p [52]. Yeast were then successfully infected with fibers grown from recombinant S35p [53]. This occurred several years before the analogous experiment was recapitulated in mammals.

Yeast Prion Structure

Like mammalian prions, fungal prions are insoluble and non-crystalline in their aggregated state, presenting many of the same obstacles to structural resolution. Despite this fact, our understanding of prion structure has progressed incrementally over the past two decades using a combination of low-resolution techniques and yeast genetics.

All known yeast prion proteins carry intrinsically disordered domains that are necessary and sufficient for prion formation [54, 55]. These domains are typically at least 80 residues long and occupy one of the terminal regions of a protein. Prion-forming domains are modular, in that they can be fused to other proteins and retain the ability to form prion [56, 57]. Likewise, the function of the non-prion domains is generally not affected by the absence of the prion-forming domain.

Yeast prion-forming domains are notable for their unusual composition. The two most-studied yeast prion domains, Ure2p 1-90 and Sup35p 1-123, are unusually rich in glutamine and asparagine, while charged and hydrophobic residues are under-represented. Both have sub-domains that are particularly rich in Q/N that play an important role in de-novo prion nucleation.

There are now multiple papers describing solid-state NMR (SSNMR) measurements of recombinant yeast prion fibers grown in vitro, including Sup35p [35, 43, 58] and Ure2p [43, 45, 46]. Yeast prions grown in vitro from recombinant protein have been demonstrated to be infectious [58]. In all of these studies, intermolecular dipole-dipole interactions among ^{13}C-labelled sites indicate that prion-forming domains aggregate into in-register parallel beta sheets when in the prion state.

In this motif, the fiber is comprised of one or more continuous beta sheets. Individual monomers run perpendicular to the axis of the fiber, with each monomer providing a single strand to the beta-sheets. This model allows the numerous glutamine and asparagine residues to line up with one another, providing stabilizing polar zippers. Indeed, structures of micro-crystals grown from a small fragment of Sup35p formed a parallel in-register beta-sheet stabilized by both backbone and side-chain hydrogen bonds [59]. Randomly rearranging residues within a prion domain does not eliminate prion-forming ability, a finding that is consistent with a parallel

in-register beta-sheet, as this motif would keep the polar zippers running along the fiber in line with one another [60, 61]. SS NMR data has been collected on some of these shuffled prion domains and the results were consistent with parallel in-register beta-sheets [43].

Alternatively, the Lindquist group proposed a "head to head, tail to tail" β-helix structure for [PSI^+] fibers based on proximity measurements of fluorescently (pyrene) labeled Sup35p [62]. The authors measured strong red-shifted excimer fluorescence, indicating pyrene group co-localization, only when labels were located in residues 25-38 and 91-106. However, the fact that pyrene malemide groups do not associate in the core 38-91 region of an amyloid fiber does not necessarily preclude the possibility of co-localization. The phenomenon of excimer fluorescence requires specific orientation of the pyrene groups. This may not be possible in the tightly packed core region of an amyloid fiber. Thus, their results may be an artifact of their experimental technique.

Yeast Prion Variants

In the early stages of mammalian prion research, scientists isolated distinct strains of scrapie infection distinguished by specific incubation times, attack rates and patterns of neuropathy [63]. Initially, these strains were taken as evidence for the hypothesis that scrapie was caused by a nucleic acid-based parasitic infectious agent. However, prion strains were subsequently demonstrated to be caused by structural differences in PrP^{sc} [31]. Yeast prions show these same structural differences, dubbed variants so as not to be confused with yeast strains. Yeast prion variants differ in the intensity and stability of the prion phenotype, and the structure and size of the protein fibers [62, 64-67]. Strong [PSI^+] strains show deep red color in the ½ YPD assay (see methods, Chapter 2 for an assay description) and are typically associated

with larger number of smaller aggregates [67]. Presumably the greater number of exposed fiber ends act as active sites recruiting a greater share of the available monomeric protein [64]. When two yeast variants are introduced to a cell, the variant that generates seeds more rapidly causes the loss of the other prion, presumably by starving it of monomer [68-70]. Yeast variants isolated in vitro showed structural differences including the locations of folds in the β-sheet [71] and differences in the length of the β-sheet region [30].

The Role of Chaperone Proteins in Yeast Prion Biology

Yeast prions were first shown to be dependent on chaperone activity by Chernoff et al. when they demonstrated that the heat-shock protein Hsp104p was required for the propagation of [PSI^+] [72]. Transient Hsp104p inhibition was subsequently shown to increase the size of prion aggregates [66, 73], a finding corroborated by live-cell imaging of yeast cells with GFP-appended Sup35p [74]. These results indicated that chaperones severed amyloid fibers into smaller "seeds" or propagons allowing for exponential amyloid growth and infection of budding daughter cells. Point mutations to the translocation channel of Hsp104p have been shown to eliminate prion propagation, hinting that it most likely operates by a substrate-threading mechanism [75, 76].

Hsp104p amyloid severing requires a supporting cast of chaperone proteins, including members of the Hsp70 and Hsp40 families, whose interactions are not fully understood. Hsp70 proteins Ssa1-4p appear to be crucial to the activation of Hsp104p. Isolation of fibrous Sup35p from [PSI+] yeast revealed high levels of *SSA1* and *SSA2* associated with Sup35p [77]. A number of point mutations to Ssa1-4p hamper or eliminate prion maintenance [78, 79]. More specifically, mutations to the substrate-binding domain of *SSA1* or *SSA2* (Ssa-21, L483W) were shown to eliminate [URE3] and [PSI^+] infections [80]. Prion maintenance was rescued in these

strains by introducing point mutations to *HSP104* (H15Y, T160M), indicating that Ssa proteins interface directly with Hsp104p. Taken together, these results indicate that Ssa1-4p probably act as recruitment proteins, localizing Hsp104p to amyloid fibers.

Conversely, the Hsp70 proteins Ssb1p and Ssb2p are believed to be [PSI^+] antagonists, binding to soluble Sup35p and preventing prion formation. Over-production of Ssb1 and 2 along with Hsp104p results in increased [PSI^+] curing efficiency, while knocking out Ssb1 and 2 increases the rate of de novo [PSI+] formation [81-83].

The Hsp40 family of co-chaperones work in conjunction with Hsp70. At least two Hsp40 proteins are necessary for the maintenance of a prion. Sis1 is known to mediate the interaction between Ssa1 and Rnq1 [84, 85], and Ydj1p has been implicated in the maintenance of the Swi1p (see the discussion about Rnq1p and Swi1p below).

The Species Barrier

Prions are known to induce aggregation in other heterologous prion proteins. For example, infected sheep brain homogenate can infect hamsters with spongiform encephalopathy despite significant differences in their PrP sequences. Typically the infection crosses this so-called 'species barrier' with a reduced rate of attack and an extended incubation period, indicating that although infectious transfer occurs, it does so with reduced efficiency. Interest in the mechanism behind this phenomenon spiked in the mid-nineties when it was discovered that consuming BSE-infected beef transmitted a variant of Creutzfeldt-Jakob disease to humans. Yeast prions have proven to be a productive model for studying the sequence requirements of heterologous prion interactions. This is due in large part to the ease with which transgenic yeast strains are generated, and the multiple experimental routes by which prion compatibility can be

investigated in a yeast system. While this subject is not the central focus of this thesis, it is something that this researcher is interested in. Indeed, in 2012 we published a review article summarizing the current research surrounding heterologous prion domain interactions [86]. This review is included as Addendum A.

Newly Discovered Prions

The discovery of yeast prions raised the exciting possibility that they may be more common phenomena than previously believed. More than fifteen years of research has resulted in the discovery of at least nine new confirmed prions and many other candidates. What follows is a brief discussion of the confirmed new yeast prions with special focus on the experimental methods used to discover them, the assay used to study them and the role their protein plays in the cell. See Figure 1.4 for a summary of this data.

[PIN$^+$]

The [PIN$^+$] prion was first discovered when researchers in the Liebman laboratory noticed that yeast cured of [PSI$^+$] carried one of two phenotypes. One, dubbed pin+, for [PSI$^+$] inducing, formed prion at rates similar to the uncured parent strain while the second, dubbed pin-, formed [PSI$^+$] at rates several orders of magnitude lower. Researchers suspected that pin+ was the result of a prion as it was dominant, non-Mendelian and reversibly curable [87]. The protein behind this phenotype was eventually identified in a bioinformatics search for candidates with high Q/N content, disorder propensity and constitutive expression, as Rnq1p [88].

Rnq1p has no known function, so there is no direct, loss-of-function assay to screen for the presence of [PIN$^+$]. However, Rnq1p aggregation in the [PIN$^+$] state was verified using GFP-fused to Rnq1p [88]. Also, transgenic strains of yeast were generated with the prion-forming

domain of Sup35 replaced by the prion-forming domain of Rnq1p. In these strains, Rnq1p prion-forming domain aggregation is monitored via the [PSI^+] phenotype [88]. Rnq1p was also purified and shown to form amyloid fibers in vitro [89].

[Het-S]

[Het-S] is unusual in a number of respects. First, it is the only prion found in the filamentous fungus *Podospora anserina*. The prion domain of Het-s is also compositionally dissimilar to the yeast prions, having Q/N content of only approximately 10%. [Het-S] is also the only prion with a well-established biological function [89]. It aids in the identification of genetically dissimilar individuals through a process called heterokaryon incompatibility. When two *P. anserina* cells come into contact with one another, their hyphae fuse and they become a single multi-nuclear organism. Heterokaryon fusion provides an avenue for viral transmission, and is discouraged by a number of mechanisms. [Het-S] provides one of those mechanisms. *P. anserina* carry two antagonistic allelic variants of the [Het-S] protein. One allele, Het-s, is capable of adopting a self-propagating alternate state, dubbed [Het-S]. The other, HET-S, is not. Fusion between a [Het-S] hypha and HET-S hypha results in localized cell death and creates a barrage of dead material that prevents further mixing of genetically dissimilar strains. The exact mechanism of [Het-S]/HET-S cytotoxicity is not well understood.

[Het-S] is the only prion whose aggregated structure has been solved at high resolution. The structure was solved through 134 unambiguous distance constraints provided by SSNMR [90], and further refined with a larger subset of ambiguous constraints [91]. In the prion state, the C-terminal domain of Het-s adopts a left-handed β-solenoid structure with a triangular hydrophobic core. Each Het-s monomer contributes two turns to the β-solenoid.

Table 1.2 A summary of prion proteins, their known functions and the yeast assay used to monitor them.

Prion	Protein	Function(s)	Yeast Assay
[URE3]	Ure2p	Transcription regulator of nitrogen uptake. Also acts as a glutathione peroxidase.	Loss-of-function assay. The ability to uptake Ureido succinate (USA) can be used to monitor [URE3]. In the presence of a good nitrogen source, Ure2p prevents the uptake of USA. Ura2Δ mutant will grow on plates spiked with USA, when Ure2p is inactivated by prion aggregation.
[PSI$^+$]	Sup35p	Translation termination factor eRF3.	There are two commonly used loss-of-function assays. 1) A nonsense mutation inserted into in the Ade1 gene results in a strain that only grows when supplied with adenine or when Sup35p is inactivated. 2) Colonies from this strain grown on ½ YPD plates are red in the [PSI+] state and white in the psi- state.
[PIN$^+$]	Rnq1p	No known function. The prion state facilitates the nucleation of other prions.	Can be detected by the dramatic increase in de novo [PSI$^+$] formation in [PIN$^+$] strains. Can also be detected by creating a chimera of the prion-forming domain of *RNQ1* and the functional domain of *SUP35*. In strains expressing this chimera, the [PSI$^+$] loss-of-function assay reports the presence of [PIN$^+$] [83].
[NU$^+$]	New1p	Function unknown. Shares homology with ABC transporter.	No direct loss-of-function assay. Can be detected by creating a chimera of the prion-forming domain of *NEW1* and the functional domain of *SUP35*. In strains expressing this chimera, the [PSI$^+$] loss-of-function assay reports the presence of [NU$^+$] [90]
[SWI$^+$]	Swi1p	Swi1p is a component of the SWI/SNF chromatin remodeling complex.	Loss-of-function assay. Screen for slow growth on carbon sources other than glucose [94].
[OCT$^+$]	Cyc8p	Cyc8p is a part of the Cyc8/Tup1 transcription co-repressor complex.	Loss-of-function assay. Iso-2 cytochrome c is overexpressed in [OCT+] cells. Yeast needs cytochome c to grow on non-fermentable carbon sources. Most of a yeast cell's cytochrome c activity comes from iso-1-cytochrome c encoded in the *CYC1* gene. So a *CYC1* knockout will not grow if lactate is the only available carbon source. [OCT+] can be isolated by screening for *CYC1* knockout strains that grow on lactate [94].
[MOT3$^+$]	Mot3p	Transcription factor.	Loss-of-function assay. Mot3p is a transcription factor that represses anaerobic metabolism when the cell is in an aerobic environment. An anaerobic gene, *DAN1* was replaced with *URA3*, allowing for [MOT3$^+$] detection by traditional uracil selection [102].
[ISP$^+$]	Sfp1p	Transcription factor.	Loss-of-function assay. Termination efficiency monitored in strains with *SUP35* mutations.
[NSI$^+$]	Vts1p	Post transcriptional regulation of mRNA.	In strains with mutations in *SUP35* or *SUP45*, the [ISP$^+$] prion restores stop-codon function [106].
[MOD$^+$]	Mod5p	tRNAisopentenyl transferase.Plays a role in tRNA gene mediated silencing.	Mod5 provides resistance to the fungicide 5-fluorouracil (5-FU), so the [MOD+] state was monitored by searching for sensitivity to 5-FU [106].
[HET-S]	Het-s	Heterokaryon incompatibility. Also identified as a selfish genetic element.	N/A

[Nu$^+$]

The New1p protein was initially flagged as a potential prion in a genome-wide bioinformatics search for glutamine/asparagine-rich domains [92]. It also turned up in a screen for proteins that induce the formation [PSI$^+$] when overexpressed [93].

New1p's function in the cell is unknown. However, it co-sediments with polysomes, and it has been suggested that it plays a role in the biogenesis of the 40S small ribosomal subunit [94]. New1p's ordered, C-terminal domain shares homology with translation elongation factors (approximately residues 131-1196).

There is no loss-of-function assay that allows for the direct monitoring of New1p aggregation. However, when the prion-forming domain of New1p (approximately residues 1-131) is appended to GFP, the resulting chimera aggregates in vivo when overexpressed [1]. It can also be attached to the C domain of Sup35, where aggregation can be monitored by the Ade assay. Its prion form, dubbed [Nu$^+$], displays all of the characteristic hallmarks of a prion, including dominant non-Mendelian inheritance, reversible curability with low concentrations of GuHCl and transmission via cytoduction. To date, there is no direct evidence that full-length New1p forms prion.

[SWI$^+$]

Swi1p was first identified as a prion candidate in bioinformatics searches for Q/N-rich domains [95]. Like a number of other prions, it was shown to behave as [PIN$^+$] when overexpressed [2].

Swi1p is a component of the 1MDa SWI/SNF chromatin remodeling complex [96]. The complex is not essential, but null mutants show phenotypes of slow growth, reduced mating-type switching, and reduced growth on non-fermentable carbon sources (i.e. raffinose, galactose, glycerol, sucrose).

The [SWI$^+$] prion was experimentally verified by the Li lab [97]. They screened for slow growth on carbon sources other than glucose to isolate [SWI$^+$]. Like other prions, [SWI$^+$] is a dominant non-Mendelian phenotype that can be transmitted via cytoduction. Also like other prions, [SWI$^+$] is mediated by chaperone proteins; [SWI$^+$] is cured by *HSP104* deletion but not by HSP104 knockout [97].

[OCT$^+$]

Cyc8p was first identified as a prion candidate in a screen for proteins that increase the rate of [PSI$^+$] formation when overexpressed [2]. Cyc8p is part of the Cyc8/Tup1 complex, a co-repressor implicated in the regulation of more than 300 genes in standard growth conditions [98, 99]. *CYC8* mutations cause slow growth, defects in sporulation and mating, elevated iso-2-cytochrome c expression, flocculation and invertase de-repression [100-102].

Patel et al. screened for aggregated Cyc8p using the increase in iso-2-cytochrome c expression that occurs upon Cyc8p sequestration [103]. Yeast needs cytochrome c to grow on non-fermentable carbon sources. Most of a yeast cell's cytochrome c activity comes from iso-1-cytochrome c, so a *CYC1* knockout will not grow if lactate is the only available carbon source. However, a cyc1 mutant can grow if iso-2-cytochrome c is overexpressed, as is the case when Cyc8p loses function, so the authors screened for *CYC8* prions by isolating *cyc1* strains that grow on lactate.

Using this assay, the authors showed that over-expression of the prion domain (residues 465-966) increased the rate of [OCT^+] prion formation by more than 100-fold [103]. The [OCT^+] phenotype was dominant and transmissible through cytogamy. The phenotype could also be cured via *HSP104* knockout, as well as by inactivation of *HSP104* using low levels of GuHCl.

[$MOT3^+$]

Mot3 is a transcription factor mediating a wide range of cellular function, including carbon metabolism, stress response and mating [104]. It was targeted as a potential prion in a survey of prion-like behaviors of 100 Q/N-rich domains with overall composition similar to known prions [105]. The candidate domains were tested for their ability to form prions using four assays: in vitro aggregation monitored via thioflavin-T binding and gel shift, in vivo aggregation monitored via fluorescent labeling, and nonsense suppression in a *SUP35*C chimera. One protein, Mot3p, was singled out for rigorous analysis, as it is amenable to a convenient loss-of-function assay.

Mot3p represses anaerobic metabolism when the cell is in an aerobic environment. The authors replaced an anaerobic gene, *DAN1* with *URA3*, allowing for prion screens via traditional uracil selection.

Using this system, the authors demonstrated that [$MOT3^+$] carries all of the typical hallmarks of a prion. The rate of [$MOT3^+$] formation is dependent on the expression level of the prion domain of [$MOT3^+$]. The [$MOT3^+$] phenotype is dominant and can be transmitted via cytoduction. Yeast cells can be infected with [$MOT3^+$] by transformation with fibers generated from recombinant protein and [$MOT3^+$] propagation is mediated by chaperone protein activity [105].

[ISP$^+$]

Sfp1p is a transcription regulation protein that mediates a wide range of biological functions including DNA-damage response [106], ribosome synthesis [107, 108], and regulation of cell size [107].

Like [URE3] and [PSI$^+$], the [ISP$^+$] phenotype was not identified in a screen for prions. It was identified as an unusual yeast phenotype before it was known to be a prion [109]. Cells carrying [ISP$^+$] show decreased levels of non-sense codon readthrough, or non-sense anti-suppression. In strains with mutations in *SUP35* or *SUP45*, the [ISP$^+$] prion restores stop-codon function.

Like other prions, the [ISP$^+$] phenotype is dominant, and can be transmitted through cytoduction. In the [ISP$^+$] state, Sfp1p forms aggregates. Interestingly aggregation occurs in the nucleus, not the cytoplasm [110]. It can also be cured with low concentrations of GuHCl. However, [ISP$^+$] is an atypical prion in a couple of key respects.

First, although [ISP$^+$] is cured by treatment with low levels of GuHCl, it is not cured by knocking out or over-expressing the *HSP104* gene [109]. This is perplexing as GuHCl is believed to cure prions by inhibiting the disaggregation activity of Hsp104p. To date, it is unclear how GuHCl acts on [ISP$^+$].

Second, the [ISP$^+$] phenotype does not resemble the *SFP1* knockout phenotype. Knocking out the *SFP1* results in decreased cell size and hypersensitivity to drugs affecting translation. [ISP$^+$] cells actually show the opposite phenotype, increased cell size and resistance to translation-impairing drugs [109].

[NSI$^+$]

[NSI$^+$] was first discovered as a nonsense-suppression phenotype that is identical to the [PSI$^+$] prion, but occurs in yeast strains that have the *SUP35* N or NM domain deleted. The [NSI$^+$] phenotype is dominant, non-Mendelian, reversibly curable, and can be transmitted by cytoduction [111]. [NSI$^+$] is also chaperone-dependent and *HSP104* inactivation cures the [NSI$^+$] phenotype.

Recently, a screen of overexpressed protein domains was used to link the [NSI$^+$] phenotype to the *VTS1* gene, which encodes a protein involved in post-transcriptional regulation of mRNA [112]. The mechanism by which *VTS1* inactivation affects stop-codon read-through is not well understood. The nonsense-suppression phenotype is only present in strains with the prion domain of Sup35 eliminated, so the phenotype is arguably not biologically relevant.

[MOD+]

Mod5 is a tRNA isopentenyltransferase that catalyzes the transfer of an isopentenyl group to A37 in the anticodon loop [113]. It is somewhat unusual in that it does not contain a Q/N rich domain like many of the other prion domains. It acts as both a PIN factor and a QIN factor. A QIN factor is any protein that induces the prion state of a Sup35 mutant where the prion-forming domain has been replaced with a 62-glutamine repeats [114].

Because Mod5 lacks a Q/N rich region, it wasn't immediately obvious where to look for the prion-forming domain. The authors found the prion-forming domain by partially digesting amyloid fibers with proteinase K and analyzing the products with mass spectrometry. The unusually short domain between residues 194 and 215 was identified as the prion-forming domain.

Mod5 is known to provide resistance to the fungicide 5-fluorouracil (5-FU), so the [MOD+] state was monitored by searching for sensitivity to 5-FU. Using this assay, the authors demonstrate that the [MOD+] state is dominant, curable by elimination of Hsp104p, and transmissible via cytoduction.

How Common are Prions?

The discovery of multiple new prions highlights a number of unanswered questions in the prion field. First, how common are prion-forming domains? The growing list of prions in a diverse array of host species hints at the intriguing possibility that prions are a widespread biological phenomenon.

The currently known yeast prions were found largely based on their compositional similarity to other prions. Bioinformatics searches for proteins with compositional similarity to Ure2p and Sup35p have been used by several groups [88, 95, 115] to flag proteins as prion candidates, and a number of these proteins were later experimentally verified as prions.

Prions are also known to induce aggregation in heterologous prion-forming domains. Derkatch et al. used this phenomenon to screen for new prions, specifically searching for proteins that induced the formation of [PSI+] in the absence of [PIN+] [2]. This approach flagged nine novel candidates, at least two of which were later verified to be prions. Heterologous prion domain interactions are more likely between compositionally similar prion domains [86], so once again, this method is biased towards prions that are compositionally similar to known prions.

These approaches have some obvious drawbacks. First, not all prion-forming domains are compositionally similar to those found on Sup35 and Ure2. The prion-forming domains of

PrP and Het-S are not particularly Q/N rich. Also, although similarity to existing prion domains has been somewhat effective at identifying prion candidates, it hasn't proven to be a good predictor of the prion propensity of a Q/N rich domain. The Lindquist group used a hidden Markov Model to list the top 100 proteins, ranked by their compositional similarity to the prion-forming domains of Sup35, Rnq1, Ure2, and New1. Four assays were used to determine the prion-forming propensity of all 100 proteins. Within this set of 100 proteins, there was almost no correlation between the similarity ranking and experimentally determined prion propensity [105].

All known naturally occurring prions are amyloids. A number of groups have designed algorithms that predict the amyloid propensity of protein with varying degrees of success [116-120]. Unfortunately, all these algorithms do a poor job of predicting prion propensity in the yeast context. This is most likely because they are designed to search for short hydrophobic stretches that drive amyloid formation in mammals. Yeast prion domains tend to feature relatively long glutamine-asparagine-rich stretches and have relatively few hydrophobic residues.

In order to better understand how yeast prion domain composition drives prion formation, Toombs et al. designed an experiment to quantify the prion propensity of each amino acid in a yeast prion [121]. An eight-residue segment in a scrambled copy of Sup35 (Sup35-27) was replaced with a random library of sequences. The library was shuffled into yeast cells, which were screened for the ability to form [PSI^+]. The prion propensity of each amino acid was determined by comparing its frequency of occurrence in the starting library to its frequency among the subset of mutants that maintained the ability to form prions. In order to verify that their results were not just an artifact of the specific location they chose, the experiment was repeated in a second location in the scrambled Sup35 prion-forming domain.

Toombs et al. found that in both locations prolines and charged residues rarely occurred in the prion-forming mutants. This was expected as they are known to discourage amyloid formation. However, it was also found that hydrophobic residues, despite being relatively rare amongst prion-forming domains, were common among prion-forming clones, indicating that they strongly promote prion formation. Also, Q/N residues, which are heavily over-represented in prion domains, were found to be neutral with respect to prion propensity.

Toombs et al. used these prion propensities to construct an algorithm for predicting the prion propensity of a given domain, dubbed PAPA for Prion Aggregation Prediction Algorithm [121, 122]. The PAPA algorithm works as follows: First, a protein is scanned using a 41-residue window, calculating the average of all prion propensities within the window. Second, the protein is re-scanned, this time calculating the average prion propensity of 41 consecutive 41-amino acid windows. Finally, this two-step process is repeated calculating the average Fold Index of each 41 amino acid window, and then the average Fold Index of 41 consecutive 41-amino acid windows. Fold Index is a freely-available, web-accessible algorithm that predicts if a given protein sequence is intrinsically disordered. It implements an algorithm developed by Uversky and co-workers, which calculates disorder propensity using the average residue hydrophobicity and net charge of a sequence [123].

Prion-forming domains are those domains that have high prion propensity and low intrinsic order. This algorithm distinguishes between Q/N-rich prion domains and Q/N-rich non-prion domains, a feat no other algorithm has accomplished.

The role of hydrophobic residues in prions is somewhat controversial. As mentioned earlier, hydrophobic residues are underrepresented in yeast prions. This would seem to suggest that they inhibit, or at least don't promote, prion formation. Indeed, this is an implicit assumption

made by any of the algorithms that search for prions using compositional similarity to existing prion domains, as they will reject a domain if its hydrophobic content is significantly higher than known prions.

Recently, Alexandrov et al. demonstrated that aromatic hydrophobic residues promote yeast prion propagation when inserted into polyglutamine tracts [124]. They demonstrated that the aromatic residues allow for more efficient chaperone-mediated fiber fragmentation. However, non-aromatic hydrophobic residues were not reported to have the same effect.

Our own data implies that adding hydrophobic residues to a yeast prion domain increases prion propensity. Chapter Two of this dissertation explores the role that hydrophobic residues play in the context of a wild-type Sup35 prion domain. In order to better understand how hydrophobic residues affect prion nucleation and amyloid growth characteristics, we use a series of yeast genetics assays and biochemical and computational techniques to verify the PAPA results in a wild-type protein. These findings were published in 2012, in PLoS One [125].

Why do prion-forming domains exist?

When they were first discovered, yeast prions were generally assumed to be biologically analogous to mammalian prion diseases. Like mammalian prion disease, their causative agent is a misfolded protein, capable of transmission from one individual to another. Also, de novo prion formation is a fairly rare event. Approximately one in a million cells carry a prion, a rate roughly comparable with de novo amyloidosis in mammals. Thus, it was reasoned that prion-forming domains either serve some function, or are vestigial domains that once served a function, and prion infections are the rare side-effect of an otherwise benign domain [126]. However, the discovery of multiple new prions, one of which, Het-S, has a well-established biological

function, coupled with the clarification of the delicate requirements of yeast prion maintenance, has drawn a number of researchers to embrace the theory that the prion state itself may provide some biological benefit [127, 128].

Prions as a disease

It has been argued that if prions are a disease, one would expect them to occur rarely in the wild, as they would present a disadvantage to their host. Early screens of wild yeast strains were unable to identify prions [129-131]. However, a large-scale screening of 690 strains revealed 10 strains that carried the [PSI+] prion (1.45%) and 43 strains that carry the [PIN] prion (6.23%). In the same paper, 96 wild yeast strains were screened for [MOT3+] and six carried the [MOT3+] prion (6.25%) [132]. These findings clearly undercut the argument that prions never occur in the wild and thus cannot be beneficial.

It has also been argued that if the prion state were a beneficial phenotype, one would expect prion-forming domains and prion activity to be conserved across closely related yeast species. To test this hypothesis, the Wickner lab looked at whether Ure2p from other yeast species were able to form prions [133]. They found that the Ure2p from *Candida albicans,* which does not conserve the *S. cerevisiae* Ure2 prion-forming domain sequence, was able to form [URE3] when cloned into *S. cerevisiae*. Conversely, the Ure2p of *Candida glabrata,* whose sequence closely resembles *S. cerevisiae,* does not. The authors interpreted this as evidence that the prion-formation is a unique fluke event, limited to *S. cerevisiae* and a few other species. However, this argument is somewhat undercut by the list of yeast species whose Sup35 prion-forming domains have been demonstrated to form prions when cloned into *S. cerevisiae*. This includes *Candida albicans*, *Pichia methanolica* and *Kluyveromyces lactis* [134]. Likewise, the

Ure2 prion domains of *Candida albicans* and *S. uvarum* behave as prions when fused to the functional domain of *S. cerevisiae* [135]. Also, it has been demonstrated that the sequence of portions of the PFDs of both Sup35 and Ure2 are conserved across a wide range of yeast species [136].

What Do Prion-forming Domains Do in Yeast?

If the yeast prion state is a disease, the prion-forming domains must serve some function, or natural selection would have eliminated them from the yeast genome. To date, the proposed non-prion functions for yeast PDFs are deeply unsatisfying. Only a few yeast PFDs have putative functions.

It has been suggested that the PFD of Ure2 may act to protect the functional domain from proteolytic breakdown [137]. Specifically, truncating the PDF of Ure2 was demonstrated to result in faster *in vivo* degradation. This researcher is skeptical. The PFD of Ure2 represents one third of its overall length. It is not surprising that eliminating one third of a protein shortens its lifespan. Eliminating a large chunk of a protein potentially externalizes a normally internal region of the protein, exposing it to proteases. Furthermore, exposing internal hydrophobic regions will predispose a protein to aggregation. One would presumably see the same result if you eliminated the first third of many other proteins. It doesn't necessarily follow that the first third of all proteins are stabilization domains.

The authors also show that the binding efficiency of Ure2p to a transcriptional co-factor is reduced when the Ure2 PFD is removed. Once again it is not surprising that eliminating part of a protein affects the function of other parts of the protein. This doesn't necessarily answer the question of what the prion-forming domain was evolutionarily optimized to do. The prion-

forming domain is a disordered region, prone to cytotoxic aggregation. There are lots of protein binding domains that do not require aggregation-prone cytotoxicity. The selective forces preserving these domains must be related to the unusual composition of the domain, or else they would be culled from the population.

There are two papers suggesting that the prion-forming domain of Sup35 may have a regulatory function other than prion formation. Both papers suggest that the intrinsically disordered prion-forming domain of Sup35 interacts directly with Pab1p [138] and Sla1p [139]. Both of these studies look for interactions between overexpressed Sup35 prion domain and a binding partner. Over-expressing the prion domain of a protein induces amyloid formation, and these amyloids are capable of incorporating full-length Sup35. Neither of these papers performed a control to eliminate this possibility.

Prions and the "Life on the Edge" Hypothesis

Tartaglia et al. illustrate an evolutionary mechanism that may explain the existence of prion-forming domains [140]. They argue that although protein aggregation is generally evolutionarily disfavored, once a protein has evolved such that it is soluble within the narrow range of normal physiological conditions, there is no selective benefit to further reduce the aggregation propensity of a protein. This would explain why most proteins are induced to aggregate by slight changes to the cellular environment. Both protein structure and expression levels have evolved to avoid aggregation, but they do so with very little margin of safety.

Tartaglia et al. plot the in vitro aggregation rate against the in vivo expression level of a number of aggregation-prone human proteins. They find an anti-correlation between the rate of expression and the rate of aggregation, indicating that the cell has evolutionarily balanced the

rate of expression against the aggregation propensity to barely avoid protein aggregation. This "Life on the edge" theory has been invoked to explain why yeast would carry domains that cause cytotoxic misfolding events. Indeed, the Ross lab recently published a paper demonstrating how non-prion Q/N rich domains can become prions with as little as two point mutations, a finding that would seem to support this hypothesis [141].

However, yeast prions are unique in a way that is inconsistent with the "life on the edge" hypothesis. Tartaglia assumes that proteins will evolve just up to the point that they don't aggregate and then stop, as there are no longer evolutionary forces further reducing their aggregation activity. Yeast prion domains aggregate spontaneously in vitro, even at low concentrations. In vivo, this aggregation is held in check by a network of heat-shock proteins in an ATP-dependent manner (see Chapter 1 for a discussion of chaperone proteins). So prion domains are not benign domains that occasionally go awry and harm the cell. The cell is continuously expending energy to prevent aggregation. If these domains didn't provide some benefit to the cell, the burden they place on the cell would cause them to be culled from the population. Interestingly, in a survey of yeast prion-forming domain polymorphisms, Lindquist et al. found at least one example of a Sup35 polymorphism in a wild-type yeast strain that did not aggregate [129]. If the Sup35 prion-forming domain is not evolutionarily optimized to form prion, one wonders why this polymorphism, or others like it, have not overtaken the entire population. This point is highlighted by the fact that as a species, yeast are quite old, on the order of hundreds of millions of years, and they reproduce fairly quickly. If prions domains are a genetic disorder, they should have been cured by natural selection by now

Prions as a Beneficial Cellular Process

Several labs have argued that prion-forming domains are conserved because the prion state itself provides some benefit to yeast [127, 128]. Proponents of this theory have suggested that prions may be evolutionary bet-hedging mechanisms. Under normal circumstances, a small, random subpopulation of yeast cells are burdened with the prion state. When the culture encounters stress, this small population provides an alternative phenotype capable of surviving until either the stress passes, or until a random polymorphism provides a more permanent adaptation to the stress. For instance, the [URE3] prion allows for the uptake of ureido succinate and other structurally similar biomolecules, regardless of the nitrogen sources that are available. If a culture of yeast were to encounter a type of stress such that the indiscriminant uptake of ureido succinate were somehow helpful, the [URE3] cells could outlive their non-prion counterparts.

Alternatively, they argue that prions may act as so called 'genetic capacitors', elements that temporarily increase phenotypic diversity in response to stress. Sup35p and Vts1p are the only prion proteins that could be assumed to act in this way, as their prion states cause random, genome wide stop-codon readthrough. This process exposes regions of the genome that are not normally expressed and thus not subjected to stringent evolutionary pressures. It is assumed that because these areas carry more genetic diversity than the genome proper, they are more likely to provide an adaptation to stress.

The data supporting these theories is somewhat disappointing. First, if prions evolved as an adaptation to stress, one would expect there to be stress conditions where the prion strain out-survives an isogenic, non-prion strain. The Lindquist lab performed a screen where [PSI+] yeast and an isogenic, non-prion strain were exposed to various stress conditions. They do report a

number of conditions where a prion strain grew faster than a non-prion strain [142]. However, the reported advantage was minor, and these conditions were out-numbered two to one by conditions were the prion strain hindered yeast growth. It is possible that the conditions that favor yeast growth occur more frequently than the growth-inhibiting conditions, thus providing a net benefit to the cell. However, this student failed to find any rationale for why one set of stresses would occur more frequently than others, and the authors did not offer any either. Furthermore, the some beneficial phenotypes were not found to be consistent across multiple strains, and some were not subsequently reproducible [142].

This result is not so surprising when you consider how prions affect the cell. Prions sequester a protein, typically partially or completely inactivating it. What are the odds that inactivating one or more of the 11 known yeast prions will benefit the cell in the face of some random stress event? Indeed, under normal growth conditions, they slow cell growth and in some cases they are lethally cytotoxic [143].

Second, the Lindquist lab also demonstrated that the rate of de novo prion formation increases under certain stress conditions [142] [144]. They argue that this is evidence that prions are an evolutionary adaptation to stress. This experiment is specious. Other protein misfolding events also increase in response to stress. Indeed, if you subject a cell to enough stress it will die and many of its proteins will misfold. It does not follow that protein misfolding is an evolutionary adaptation to cell death.

The genetic capacitor theory also deserves closer scrutiny. Random, intermittent, proteome-wide stop-codon read-through is unlikely to create a phenotype that mitigates environmental stress. Stop-codon read-through results in proteins being appended with a random stretch of amino acids. Suppose a specific read-through event did produce a new translation

product that just happens to serve some useful new function. In order to benefit the cell, that function would have to compensate for the detrimental effects of all of the other random read-through events occurring in the cell. This seems highly unlikely. It has been suggested that the regions immediately after all stop codons in the yeast genome are expressed often enough to also be under selective pressure. To date, there is no experimental data to support this view.

Functional Amyloids

There are a number of examples of species that have co-opted the amyloid structure for the benefit of the cell. These are not necessarily prions, as they are not infectious. Rather, these are species using amyloid like any structural motif. What follows is a brief review of several known functional amyloids.

Intra cellular viral immune response

Zhijian Chen's group demonstrated that the cell's intracellular viral immune response may take advantage of the prion-like properties on a mitochondrial protein, MAVS (mitochondrial antiviral signaling protein) [145]. In response to viral infection, MAVS is induced to aggregate into large, prion like, protease resistant aggregates. The authors demonstrated that, in-vitro, MAVS aggregates are capable of recruiting soluble MAVS from cytoplasm, much like a prion. These aggregates have been linked to the dimerization and activation of the viral response transcription factor, IRF3, initiating the antiviral signaling cascade. The exact mechanism that initiates aggregation, and the way that these aggregates activate IRF3 are not yet well understood.

Prokaryotic Biofilms

A number of gram-positive bacteria, including *Escherichia coli* and *Salmonella enterica,* protect themselves by forming extracellular biofilms comprised of a hydrated mix of proteins, nucleic acids and polysaccharides. The bulk of the proteinaceous component of biofilm is made up of amyloid fibers called curli. Like many other amyloids, the exact structure of curli is unknown. However, X-ray fiber diffraction, SSNMR and electron microscopy data on curli fibers grown *in vitro* indicate that curli are comprised of stacked β-helical subunits [44]. When grown in vitro, curli show growth kinetics comparable to yeast prion proteins. Following an extended lag time, in vitro fiber growth occurs at an exponential rate. The lag time can be eliminated by introducing pre-formed curli fibers [146]. Curli are hetero-polymeric filaments made up predominantly of two proteins CsgA and CsgB. CsgA is the dominant component of curli, comprising more than 95% of the fiber subunits [147]. The amino acid sequence of Csga is comprised of three domains, a signal peptide sequence, an N-terminal targeting sequence and a C-terminal amyloid sequence. The signal peptide sequence is cleaved off during intra-cellular processing, and the remainder of the protein is secreted to the cell surface [148]. CsgB has the same three-domain structure as CsgA. The CsgB protein also spontaneously form amyloid in vitro, with a significantly shorter lag time than CsgA [149].

Stress Granules (Tia1p)

TIA-1 is another RNA-binding protein that is capable of aggregating into an amyloid fiber. TIA-1 induces translational arrest and promotes the formation of stress granules, dense particles of aggregated mRNA and proteins that appear when the cell is under stress [150]. TIA-1

contains a prion-like domain at its C-terminus (residues 290-387) that exhibits many of the same characteristics of yeast prions [151].

Specifically, TIA-1 aggregates in a concentration-dependent manner. This aggregation is mediated by chaperone proteins. TIA-1 aggregation is inhibited by the recombinant expression Hsp70. Remarkably, replacing the prion like domain of TIA-1 with the prion-forming domain of the yeast protein Sup35 protein restored stress granule formation in COS-7 cells.

Yeast prion domains may also be functional amyloids, domains whose aggregation serves a biological function, but are not necessarily prions.

Unfortunately, many of the existing techniques for studying yeast prions are reliant on the fact that prions are genetic elements, passed from mother to daughter cells. Indeed, many yeast genetics assays require 3-7 days of growth before a result is available. A colony large enough to be visible to the naked eye is comprised of approximately 1-10 million yeast cells. So in order to detect a prion it has to be stable through at least 20 cell divisions. If prion-forming domains were evolved to aggregate transiently, in response to some stimulus, these assays would most likely be unable to detect that phenomenon.

Chapter 3 of this dissertation focuses on the reasons that yeast cells form prions. Computational methods are used to explore the prevalence of QN-rich domains in a large set of species. We use this data to better understand how selective forces have preserved these domains. Also, Chapter 3 describes progress towards the development of a fluorescence-based assay, designed to detect prion domain aggregation in yeast cells.

BIBLIOGRAPHY

1. Stamp, J.T., et al., *Further studies on scrapie.* J Comp Pathol, 1959. **69**: p. 268-80.
2. Hadlow, W.J., *Myopathies of livestock.* Lab Invest, 1959. **8**: p. 1478-98.
3. Gajdusek, D.C. and V. Zigas, *Degenerative disease of the central nervous system in New Guinea; the endemic occurrence of kuru in the native population.* N Engl J Med, 1957. **257**(20): p. 974-8.
4. Prusiner, S.B., *Molecular biology and genetics of prion diseases.* Cold Spring Harb Symp Quant Biol, 1996. **61**: p. 473-93.
5. Aiken, J.M. and R.F. Marsh, *The search for scrapie agent nucleic acid.* Microbiol Rev, 1990. **54**(3): p. 242-6.
6. Pattison, I.H., *Resistance of the Scrapie Agent to Formalin.* J Comp Pathol, 1965. **75**: p. 159-64.
7. Alper, T., D.A. Haig, and M.C. Clarke, *The exceptionally small size of the scrapie agent.* Biochem Biophys Res Commun, 1966. **22**(3): p. 278-84.
8. Bellinger-Kawahara, C., et al., *Purified scrapie prions resist inactivation by procedures that hydrolyze, modify, or shear nucleic acids.* Virology, 1987. **160**(1): p. 271-4.
9. Griffith, J.S., *Self-replication and scrapie.* Nature, 1967. **215**(5105): p. 1043-4.
10. Bolton, D.C., M.P. McKinley, and S.B. Prusiner, *Identification of a protein that purifies with the scrapie prion.* Science, 1982. **218**(4579): p. 1309-11.
11. Prusiner, S.B., et al., *Further purification and characterization of scrapie prions.* Biochemistry, 1982. **21**(26): p. 6942-50.
12. Chesebro, B., et al., *Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain.* Nature, 1985. **315**(6017): p. 331-3.
13. Oesch, B., et al., *A cellular gene encodes scrapie PrP 27-30 protein.* Cell, 1985. **40**(4): p. 735-46.
14. Bueler, H., et al., *Mice devoid of PrP are resistant to scrapie.* Cell, 1993. **73**(7): p. 1339-47.
15. McKinley, M.P. and S.B. Prusiner, *Biology and structure of scrapie prions.* Int Rev Neurobiol, 1986. **28**: p. 1-57.
16. Kocisko, D.A., et al., *Cell-free formation of protease-resistant prion protein.* Nature, 1994. **370**(6489): p. 471-4.
17. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. **411**(6839): p. 810-3.
18. Castilla, J., et al., *In vitro generation of infectious scrapie prions.* Cell, 2005. **121**(2): p. 195-206.
19. Deleault, N.R., et al., *Formation of native prions from minimal components in vitro.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9741-6.
20. Wang, F., et al., *Generating a prion with bacterially expressed recombinant prion protein.* Science, 2010. **327**(5969): p. 1132-5.
21. Manuelidis, L., *A 25 nm virion is the likely cause of transmissible spongiform encephalopathies.* J Cell Biochem, 2007. **100**(4): p. 897-915.
22. Manuelidis, L., *Nuclease resistant circular DNAs copurify with infectivity in scrapie and CJD.* J Neurovirol, 2011. **17**(2): p. 131-45.

23. Wuthrich, K. and R. Riek, *Three-dimensional structures of prion proteins.* Adv Protein Chem, 2001. **57**: p. 55-82.
24. Riek, R., et al., *NMR structure of the mouse prion protein domain PrP(121-231).* Nature, 1996. **382**(6587): p. 180-2.
25. Toyama, B.H., et al., *The structural basis of yeast prion strain variants.* Nature, 2007. **449**(7159): p. 233-7.
26. Collinge, J. and A.R. Clarke, *A general model of prion strains and their pathogenicity.* Science, 2007. **318**(5852): p. 930-6.
27. McKinley, M.P., D.C. Bolton, and S.B. Prusiner, *A protease-resistant protein is a structural component of the scrapie prion.* Cell, 1983. **35**(1): p. 57-62.
28. Cronier, S., et al., *Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin.* Biochem J, 2008. **416**(2): p. 297-305.
29. Lawson, V.A., et al., *N-terminal truncation of prion protein affects both formation and conformation of abnormal protease-resistant prion protein generated in vitro.* J Biol Chem, 2001. **276**(38): p. 35265-71.
30. Shewmaker, F., R.B. Wickner, and R. Tycko, *Amyloid of the prion domain of Sup35p has an in-register parallel beta-sheet structure.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19754-9.
31. Peretz, D., et al., *A conformational transition at the N terminus of the prion protein features in formation of the scrapie isoform.* J Mol Biol, 1997. **273**(3): p. 614-22.
32. Pan, K.M., et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-6.
33. Caughey, B.W., et al., *Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy.* Biochemistry, 1991. **30**(31): p. 7672-80.
34. Eanes, E.D. and G.G. Glenner, *X-ray diffraction studies on amyloid filaments.* J Histochem Cytochem, 1968. **16**(11): p. 673-7.
35. Wille, H., et al., *Natural and synthetic prion structure from X-ray fiber diffraction.* Proc Natl Acad Sci U S A, 2009. **106**(40): p. 16990-5.
36. Govaerts, C., et al., *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A, 2004. **101**(22): p. 8342-7.
37. Cobb, N.J., et al., *Molecular architecture of human prion protein amyloid: a parallel, in-register beta-structure.* Proc Natl Acad Sci U S A, 2007. **104**(48): p. 18946-51.
38. Shewmaker, F., et al., *Amyloids of shuffled prion domains that form prions have a parallel in-register beta-sheet structure.* Biochemistry, 2008. **47**(13): p. 4000-7.
39. Shewmaker, F., et al., *The functional curli amyloid is not based on in-register parallel beta-sheet structure.* J Biol Chem, 2009. **284**(37): p. 25065-76.
40. Baxa, U., et al., *Characterization of beta-sheet structure in Ure2p1-89 yeast prion fibrils by solid-state nuclear magnetic resonance.* Biochemistry, 2007. **46**(45): p. 13149-62.
41. Kryndushkin, D.S., R.B. Wickner, and R. Tycko, *The core of Ure2p prion fibrils is formed by the N-terminal segment in a parallel cross-beta structure: evidence from solid-state NMR.* J Mol Biol, 2011. **409**(2): p. 263-77.
42. Cox, B.S., *A recessive lethal super-suppressor mutation in yeast and other psi phenomena.* Heredity (Edinb), 1971. **26**(2): p. 211-32.
43. Lacroute, F., *Non-Mendelian mutation allowing ureidosuccinic acid uptake in yeast.* J Bacteriol, 1971. **106**(2): p. 519-22.

44. Drillien, R. and F. Lacroute, *Ureidosuccinic acid uptake in yeast and some aspects of its regulation.* J Bacteriol, 1972. **109**(1): p. 203-8.
45. Wickner, R.B., *[URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae.* Science, 1994. **264**(5158): p. 566-9.
46. King, C.Y., et al., *Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6618-22.
47. Taylor, K.L., et al., *Prion domain initiation of amyloid formation in vitro from native Ure2p.* Science, 1999. **283**(5406): p. 1339-43.
48. Sparrer, H.E., et al., *Evidence for the prion hypothesis: induction of the yeast [PSI+] factor by in vitro- converted Sup35 protein.* Science, 2000. **289**(5479): p. 595-9.
49. Masison, D.C. and R.B. Wickner, *Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells.* Science, 1995. **270**(5233): p. 93-5.
50. Ter-Avanesyan, M.D., et al., *The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast Saccharomyces cerevisiae.* Genetics, 1994. **137**(3): p. 671-6.
51. Li, L. and S. Lindquist, *Creating a protein-based element of inheritance.* Science, 2000. **287**(5453): p. 661-4.
52. Baxa, U., et al., *Mechanism of inactivation on prion conversion of the Saccharomyces cerevisiae Ure2 protein.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5253-60.
53. Shewmaker, F., et al., *Two prion variants of Sup35p have in-register parallel beta-sheet structures, independent of hydration.* Biochemistry, 2009. **48**(23): p. 5074-82.
54. Nelson, R., et al., *Structure of the cross-beta spine of amyloid-like fibrils.* Nature, 2005. **435**(7043): p. 773-8.
55. Ross, E.D., et al., *Primary sequence independence for prion formation.* Proc Natl Acad Sci U S A, 2005. **102**(36): p. 12825-30.
56. Ross, E.D., U. Baxa, and R.B. Wickner, *Scrambled prion domains form prions and amyloid.* Mol Cell Biol, 2004. **24**(16): p. 7206-13.
57. Krishnan, R. and S.L. Lindquist, *Structural insights into a yeast prion illuminate nucleation and strain diversity.* Nature, 2005. **435**(7043): p. 765-72.
58. Fraser, H. and A.G. Dickinson, *Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation.* J Comp Pathol, 1973. **83**(1): p. 29-40.
59. Derkatch, I.L., et al., *Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae.* Genetics, 1996. **144**(4): p. 1375-86.
60. Uptain, S.M., et al., *Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion.* EMBO J, 2001. **20**(22): p. 6236-45.
61. Kryndushkin, D.S., et al., *Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104.* J Biol Chem, 2003. **278**(49): p. 49636-43.
62. Tanaka, M., et al., *Conformational variations in an infectious protein determine prion strain differences.* Nature, 2004. **428**(6980): p. 323-8.
63. Bradley, M.E., et al., *Interactions among prions and prion "strains" in yeast.* Proc Natl Acad Sci U S A, 2002. **99 Suppl 4**: p. 16392-9.
64. Tanaka, M., et al., *The physical basis of how prion conformations determine strain phenotypes.* Nature, 2006. **442**(7102): p. 585-9.
65. Bagriantsev, S. and S.W. Liebman, *Specificity of prion assembly in vivo. [PSI+] and [PIN+] form separate structures in yeast.* J Biol Chem, 2004. **279**(49): p. 51042-8.

66. Wickner, R.B., et al., *Prion amyloid structure explains templating: how proteins can be genes.* FEMS Yeast Res, 2010. **10**(8): p. 980-91.
67. Chernoff, Y.O., et al., *Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+].* Science, 1995. **268**(5212): p. 880-4.
68. Ferreira, P.C., et al., *The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation.* Mol Microbiol, 2001. **40**(6): p. 1357-69.
69. Satpute-Krishnan, P., S.X. Langseth, and T.R. Serio, *Hsp104-dependent remodeling of prion complexes mediates protein-only inheritance.* PLoS Biol, 2007. **5**(2): p. e24.
70. Hung, G.C. and D.C. Masison, *N-terminal domain of yeast Hsp104 chaperone is dispensable for thermotolerance and prion propagation but necessary for curing prions by Hsp104 overexpression.* Genetics, 2006. **173**(2): p. 611-20.
71. Tessarz, P., A. Mogk, and B. Bukau, *Substrate threading through the central pore of the Hsp104 chaperone as a common mechanism for protein disaggregation and prion propagation.* Mol Microbiol, 2008. **68**(1): p. 87-97.
72. Bagriantsev, S.N., et al., *Variant-specific [PSI+] infection is transmitted by Sup35 polymers within [PSI+] aggregates with heterogeneous protein composition.* Mol Biol Cell, 2008. **19**(6): p. 2433-43.
73. Hines, J.K., et al., *Influence of prion variant and yeast strain variation on prion-molecular chaperone requirements.* Prion, 2011. **5**(4): p. 238-44.
74. Jones, G.W., Y. Song, and D.C. Masison, *Deletion of the Hsp70 chaperone gene SSB causes hypersensitivity to guanidine toxicity and curing of the [PSI+] prion by increasing guanidine uptake in yeast.* Mol Genet Genomics, 2003. **269**(3): p. 304-11.
75. Jung, G., et al., *A role for cytosolic hsp70 in yeast [PSI(+)] prion propagation and [PSI(+)] as a cellular stress.* Genetics, 2000. **156**(2): p. 559-70.
76. Chacinska, A., et al., *Ssb1 chaperone is a [PSI+] prion-curing factor.* Curr Genet, 2001. **39**(2): p. 62-7.
77. Chernoff, Y.O., et al., *Evidence for a protein mutator in yeast: role of the Hsp70-related chaperone ssb in formation, stability, and toxicity of the [PSI] prion.* Mol Cell Biol, 1999. **19**(12): p. 8103-12.
78. Kushnirov, V.V., et al., *Chaperones that cure yeast artificial [PSI+] and their prion-specific effects.* Curr Biol, 2000. **10**(22): p. 1443-6.
79. Aron, R., et al., *J-protein co-chaperone Sis1 required for generation of [RNQ+] seeds necessary for prion propagation.* EMBO J, 2007. **26**(16): p. 3794-803.
80. Sondheimer, N., et al., *The role of Sis1 in the maintenance of the [RNQ+] prion.* EMBO J, 2001. **20**(10): p. 2435-42.
81. Gonzalez Nelson, A.C. and E.D. Ross, *Interactions between non-identical prion proteins.* Semin Cell Dev Biol, 2011. **22**(5): p. 437-43.
82. Derkatch, I.L., et al., *Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae.* Genetics, 1997. **147**(2): p. 507-19.
83. Sondheimer, N. and S. Lindquist, *Rnq1: an epigenetic modifier of protein function in yeast.* Mol Cell, 2000. **5**(1): p. 163-72.
84. Saupe, S.J., *The [Het-s] prion of Podospora anserina and its role in heterokaryon incompatibility.* Semin Cell Dev Biol, 2011. **22**(5): p. 460-8.

85. Wasmer, C., et al., *Amyloid fibrils of the HET-s(218-289) prion form a beta solenoid with a triangular hydrophobic core.* Science, 2008. **319**(5869): p. 1523-6.
86. Lange, A., et al., *A combined solid-state NMR and MD characterization of the stability and dynamics of the HET-s(218-289) prion in its amyloid conformation.* Chembiochem, 2009. **10**(10): p. 1657-65.
87. Santoso, A., et al., *Molecular basis of a yeast prion species barrier.* Cell, 2000. **100**(2): p. 277-88.
88. Derkatch, I.L., et al., *Dependence and independence of [PSI(+)] and [PIN(+)]: a two-prion system in yeast?* EMBO J, 2000. **19**(9): p. 1942-52.
89. Li, Z., et al., *Rational extension of the ribosome biogenesis pathway using network-guided genetics.* PLoS Biol, 2009. **7**(10): p. e1000213.
90. Osherovich, L.Z. and J.S. Weissman, *Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion.* Cell, 2001. **106**(2): p. 183-94.
91. Michelitsch, M.D. and J.S. Weissman, *A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 11910-5.
92. Derkatch, I.L., et al., *Prions affect the appearance of other prions: the story of [PIN(+)].* Cell, 2001. **106**(2): p. 171-82.
93. Smith, C.L., et al., *Structural analysis of the yeast SWI/SNF chromatin remodeling complex.* Nat Struct Biol, 2003. **10**(2): p. 141-5.
94. Du, Z., et al., *Newly identified prion linked to the chromatin-remodeling factor Swi1 in Saccharomyces cerevisiae.* Nat Genet, 2008. **40**(4): p. 460-5.
95. DeRisi, J.L., V.R. Iyer, and P.O. Brown, *Exploring the metabolic and genetic control of gene expression on a genomic scale.* Science, 1997. **278**(5338): p. 680-6.
96. Green, S.R. and A.D. Johnson, *Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in Saccharomyces cerevisiae.* Mol Biol Cell, 2004. **15**(9): p. 4191-202.
97. Smith, R.L. and A.D. Johnson, *Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes.* Trends Biochem Sci, 2000. **25**(7): p. 325-30.
98. Rothstein, R.J. and F. Sherman, *Genes affecting the expression of cytochrome c in yeast: genetic mapping and genetic interactions.* Genetics, 1980. **94**(4): p. 871-89.
99. Neigeborn, L. and M. Carlson, *Genes affecting the regulation of SUC2 gene expression by glucose repression in Saccharomyces cerevisiae.* Genetics, 1984. **108**(4): p. 845-58.
100. Patel, B.K., J. Gavin-Smyth, and S.W. Liebman, *The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion.* Nat Cell Biol, 2009. **11**(3): p. 344-9.
101. Grishin, A.V., et al., *Mot3, a Zn finger transcription factor that modulates gene expression and attenuates mating pheromone signaling in Saccharomyces cerevisiae.* Genetics, 1998. **149**(2): p. 879-92.
102. Alberti, S., et al., *A systematic survey identifies prions and illuminates sequence features of prionogenic proteins.* Cell, 2009. **137**(1): p. 146-58.
103. Xu, Z. and D. Norris, *The SFP1 gene product of Saccharomyces cerevisiae regulates G2/M transitions during the mitotic cell cycle and DNA-damage response.* Genetics, 1998. **150**(4): p. 1419-28.
104. Jorgensen, P., et al., *A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size.* Genes Dev, 2004. **18**(20): p. 2491-505.

105. Marion, R.M., et al., *Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression.* Proc Natl Acad Sci U S A, 2004. **101**(40): p. 14315-22.
106. Volkov, K.V., et al., *Novel non-Mendelian determinant involved in the control of translation accuracy in Saccharomyces cerevisiae.* Genetics, 2002. **160**(1): p. 25-36.
107. Rogoza, T., et al., *Non-Mendelian determinant [ISP+] in yeast is a nuclear-residing prion form of the global transcriptional regulator Sfp1.* Proc Natl Acad Sci U S A, 2010. **107**(23): p. 10573-7.
108. Saifitdinova, A.F., et al., *[NSI (+)]: a novel non-Mendelian nonsense suppressor determinant in Saccharomyces cerevisiae.* Curr Genet, 2010. **56**(5): p. 467-78.
109. Nizhnikov, A.A., et al., *[NSI+] determinant has a pleiotropic phenotypic manifestation that is modulated by SUP35, SUP45, and VTS1 genes.* Curr Genet, 2012. **58**(1): p. 35-47.
110. Dihanich, M.E., et al., *Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of Saccharomyces cerevisiae.* Mol Cell Biol, 1987. **7**(1): p. 177-84.
111. Suzuki, G., N. Shimazu, and M. Tanaka, *A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress.* Science, 2012. **336**(6079): p. 355-9.
112. Harrison, P.M. and M. Gerstein, *A method to assess compositional bias in biological sequences and its application to prion-like glutamine/asparagine-rich domains in eukaryotic proteomes.* Genome Biol, 2003. **4**(6): p. R40.
113. Bryan, A.W., Jr., et al., *BETASCAN: probable beta-amyloids identified by pairwise probabilistic analysis.* PLoS Comput Biol, 2009. **5**(3): p. e1000333.
114. Fernandez-Escamilla, A.M., et al., *Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins.* Nat Biotechnol, 2004. **22**(10): p. 1302-6.
115. Tartaglia, G.G. and M. Vendruscolo, *The Zyggregator method for predicting protein aggregation propensities.* Chem Soc Rev, 2008. **37**(7): p. 1395-401.
116. Zibaee, S., et al., *A simple algorithm locates beta-strands in the amyloid fibril core of alpha-synuclein, Abeta, and tau using the amino acid sequence alone.* Protein Sci, 2007. **16**(5): p. 906-18.
117. Trovato, A., F. Seno, and S.C. Tosatto, *The PASTA server for protein aggregation prediction.* Protein Eng Des Sel, 2007. **20**(10): p. 521-3.
118. Toombs, J.A., B.R. McCarty, and E.D. Ross, *Compositional determinants of prion formation in yeast.* Mol Cell Biol, 2010. **30**(1): p. 319-32.
119. Ross, E.D. and J.A. Toombs, *The effects of amino acid composition on yeast prion formation and prion domain interactions.* Prion, 2010. **4**(2): p. 60-5.
120. Uversky, V.N., J.R. Gillespie, and A.L. Fink, *Why are "natively unfolded" proteins unstructured under physiologic conditions?* Proteins, 2000. **41**(3): p. 415-27.
121. Alexandrov, A.I., et al., *The effects of amino acid composition of glutamine-rich domains on amyloid formation and fragmentation.* PLoS One, 2012. **7**(10): p. e46458.
122. Gonzalez Nelson, A.C., et al., *Increasing prion propensity by hydrophobic insertion.* PLoS One, 2014. **9**(2): p. e89286.
123. Wickner, R.B., et al., *The yeast prions [PSI+] and [URE3] are molecular degenerative diseases.* Prion, 2011. **5**(4): p. 258-62.
124. Halfmann, R. and S. Lindquist, *Epigenetics in the extreme: prions and the inheritance of environmentally acquired traits.* Science, 2010. **330**(6004): p. 629-32.

125. Halfmann, R., S. Alberti, and S. Lindquist, *Prions, protein homeostasis, and phenotypic diversity.* Trends Cell Biol, 2010. **20**(3): p. 125-33.
126. Resende, C.G., et al., *Prion protein gene polymorphisms in Saccharomyces cerevisiae.* Mol Microbiol, 2003. **49**(4): p. 1005-17.
127. Chernoff, Y.O., et al., *Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein.* Mol Microbiol, 2000. **35**(4): p. 865-76.
128. Nakayashiki, T., et al., *Yeast prions [URE3] and [PSI+] are diseases.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10575-80.
129. Halfmann, R., et al., *Prions are a common mechanism for phenotypic inheritance in wild yeasts.* Nature, 2012. **482**(7385): p. 363-8.
130. Edskes, H.K., et al., *Prion-forming ability of Ure2 of yeasts is not evolutionarily conserved.* Genetics, 2011. **188**(1): p. 81-90.
131. Kushnirov, V.V., et al., *Prion properties of the Sup35 protein of yeast Pichia methanolica.* EMBO J, 2000. **19**(3): p. 324-31.
132. Baudin-Baillieu, A., et al., *Conservation of the prion properties of Ure2p through evolution.* Mol Biol Cell, 2003. **14**(8): p. 3449-58.
133. Harrison, L.B., et al., *Evolution of budding yeast prion-determinant sequences across diverse fungi.* J Mol Biol, 2007. **368**(1): p. 273-82.
134. Shewmaker, F., et al., *Ure2p function is enhanced by its prion domain in Saccharomyces cerevisiae.* Genetics, 2007. **176**(3): p. 1557-65.
135. Hosoda, N., et al., *Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation.* J Biol Chem, 2003. **278**(40): p. 38287-91.
136. Bailleul, P.A., et al., *Genetic study of interactions between the cytoskeletal assembly protein sla1 and prion-forming domain of the release factor Sup35 (eRF3) in Saccharomyces cerevisiae.* Genetics, 1999. **153**(1): p. 81-94.
137. Tartaglia, G.G., et al., *Life on the edge: a link between gene expression levels and aggregation rates of human proteins.* Trends Biochem Sci, 2007. **32**(5): p. 204-6.
138. Paul, K.R. and E.D. Ross, *Controlling the prion propensity of glutamine/asparagine-rich proteins.* Prion, 2015. **9**(5): p. 347-54.
139. True, H.L. and S.L. Lindquist, *A yeast prion provides a mechanism for genetic variation and phenotypic diversity.* Nature, 2000. **407**(6803): p. 477-83.
140. McGlinchey, R.P., D. Kryndushkin, and R.B. Wickner, *Suicidal [PSI+] is a lethal yeast prion.* Proc Natl Acad Sci U S A, 2011. **108**(13): p. 5337-41.
141. Tyedmers, J., M.L. Madariaga, and S. Lindquist, *Prion switching in response to environmental stress.* PLoS Biol, 2008. **6**(11): p. e294.
142. Hou, F., et al., *MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response.* Cell, 2011. **146**(3): p. 448-61.
143. Wang, X., et al., *In vitro polymerization of a functional Escherichia coli amyloid protein.* J Biol Chem, 2007. **282**(6): p. 3713-9.
144. White, A.P., et al., *Structure and characterization of AgfB from Salmonella enteritidis thin aggregative fimbriae.* J Mol Biol, 2001. **311**(4): p. 735-49.
145. Chapman, M.R., et al., *Role of Escherichia coli curli operons in directing amyloid fiber formation.* Science, 2002. **295**(5556): p. 851-5.
146. Shu, Q., et al., *The E. coli CsgB nucleator of curli assembles to beta-sheet oligomers that alter the CsgA fibrillization mechanism.* Proc Natl Acad Sci U S A, 2012. **109**(17): p. 6502-7.

147. Anderson, P. and N. Kedersha, *Visibly stressed: the role of eIF2, TIA-1, and stress granules in protein translation.* Cell Stress Chaperones, 2002. **7**(2): p. 213-21.
148. Gilks, N., et al., *Stress granule assembly is mediated by prion-like aggregation of TIA-1.* Mol Biol Cell, 2004. **15**(12): p. 5383-98.

CHAPTER 2: THE EFFECTS OF HYDROPHOBICITY ON YEAST PRION FORMATION

Adapted From:

Increasing prion propensity by hydrophobic insertion

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Introduction

Prion formation involves the conversion of proteins from a soluble form into an infectious amyloid form. Most yeast prion proteins contain glutamine/asparagine-rich regions that are responsible for prion aggregation. Prion formation by these domains is driven primarily by amino acid composition, not primary sequence, yet there is a surprising disconnect between the amino acids thought to have the highest aggregation propensity and those that are actually found in yeast prion domains. Specifically, a recent mutagenic screen suggested that both aromatic and non-aromatic hydrophobic residues strongly promote prion formation. However, while aromatic residues are common in yeast prion domains, non-aromatic hydrophobic residues are strongly under-represented. Here, we directly test the effects of hydrophobic and aromatic residues on prion formation. Remarkably, we found that insertion of as few as two hydrophobic residues resulted in a multiple orders-of-magnitude increase in prion formation, and significant acceleration of *in vitro* amyloid formation. Thus, insertion or deletion of hydrophobic residues

provides a simple tool to control the prion activity of a protein. These data, combined with bioinformatics analysis, suggest a limit on the number of strongly prion-promoting residues tolerated in glutamine/asparagine-rich domains. This limit may explain the under-representation of non-aromatic hydrophobic residues in yeast prion domains. Prion activity requires not only that a protein be able to form prion fibers, but also that these fibers be cleaved to generate new independently-segregating aggregates to offset dilution by cell division. Recent studies suggest that aromatic residues, but not non-aromatic hydrophobic residues, support the fiber cleavage step. Therefore, we propose that while both aromatic and non-aromatic hydrophobic residues promote prion formation, aromatic residues are favored in yeast prion domains because they serve a dual function, promoting both prion formation and chaperone-dependent prion propagation.

Background

Prions are protein-based infectious agents, caused by proteins capable of adopting an alternative, self-propagating amyloid-like structure. In mammals, misfolding of the prion protein PrP is responsible for the transmissible spongiform encephalopathies (TSEs), all of which are incurable and fatal [152]. Additionally, many other non-infectious diseases also involve the aggregation of proteins into amyloid deposits. In fungi, a number of proteins can adopt a prion state. The filamentous fungus *P. anserine* carries a prion protein, Het-S [153], that acts as part of a heterokaryon incompatibility mechanism. The yeast *Saccharomyces cerevisiae* carries at least nine proteins that convert to a prion state [154].

Yeast prions provide a useful model system for examining how amino acid sequence affects amyloid and prion propensity. For all but one of the amyloid-based yeast prion proteins,

a glutamine/asparagine (Q/N) rich prion-forming domain (PFD) drives prion formation. Intriguingly, in the past few years, a number of proteins with prion-like domains (domains compositionally resembling the yeast PFDs) have been linked to various age-related degenerative disorders [155]: cytoplasmic inclusions containing FUS and TDP-43 are seen in both ALS and some forms of FTLD, and mutations in these proteins have been linked to some familial cases of ALS [156-158]; TAF15 and EWSR1 have separately been connected to ALS and FTLD [159-161]; mutations in hnRNPA1 and hnRNPA2/B1 cause IBMPFD/ALS (inclusion body myopathy with frontotemporal dementia, Paget's disease of bone, and ALS; [162]); and mutations in TIA1 cause Welander distal myopathy [163]. A better understanding of how sequence and composition affect the amyloid propensity of prion-like domains would permit a better understanding of the mechanism of aggregation in these diseases. It would also allow for bioinformatics searches to identify new prion-like domains.

The yeast prion protein Sup35, which forms the [*PSI*$^{+}$] prion, is an essential subunit of the translation termination complex. Sup35 has three functionally distinct domains [55, 164, 165]. The N-terminal PFD (residues 1-114) is an intrinsically disordered domain that is necessary and sufficient for prion aggregation [55, 164, 165]. Like other yeast prions, it has high Q/N content and few hydrophobic residues [166]. The M domain (residues 114-253) is a highly charged, intrinsically disordered region that is not required for either prion formation or translation termination activity, but that stabilizes [*PSI*$^{+}$] [167]. The C domain (residues 253-685) is a structured region that is necessary and sufficient for translation termination.

Scrambling the PFD of Sup35 does not prevent prion formation, demonstrating that composition is a dominant variable affecting prion propensity [168]. A number of search algorithms to identify new prion proteins have been developed that take advantage of this fact by

testing for compositional similarity to known PFDs [105, 166, 169]. Several prions were discovered using these methods [105, 170, 171]. However, this approach has limitations. Alberti *et al*. identified 100 yeast domains that had the greatest compositional similarity to existing PFDs and tested them for amyloid and prion-like activity using four different assays [105]. Remarkably, eighteen behaved as prions in all four assays. However, there was almost no correlation between the prion-forming ability of the 100 tested domains and their compositional similarity to existing PFDs [122, 172]. Therefore, while this algorithm is very effective at identifying prion candidates, it was ineffective at distinguishing among these candidates.

To better understand how composition affects prion propensity, we developed a method to quantify the prion-forming propensity of each amino acid in the context of a Q/N-rich PFD [172]. We replaced an eight amino acid segment from a scrambled Sup35 with a random library of sequences. We then selected for the subset of sequences that could form prions; the prion propensity of each amino acid was determined by comparing the frequency of occurrence of the amino acid among the prion-forming sequences to the frequency of the amino acid in the starting library. These prion propensity values were then used to build the prediction algorithm PAPA (Prion Aggregation Prediction Algorithm; [173, 174]).

PAPA is quite effective at discriminating between Q/N-rich domains with and without prion activity [172]. However, some of the individual prion propensity values for specific amino acids were quite surprising. As expected, charged residues and prolines were under-represented among prion-forming clones, consistent with their relative rarity in yeast PFDs [172]. Unexpectedly, Q/N residues were relatively neutral despite their prevalence in yeast PFDs, while hydrophobic residues, which are rare in yeast PFDs [166], were strongly over-represented among prion-forming clones, suggesting that they strongly promote prion activity.

This high predicted prion propensity for hydrophobic residues is particularly intriguing. The strong under-representation of hydrophobic residues in yeast PFDs would seem to suggest that these residues inhibit prion activity in the context of Q/N-rich domains. Indeed, any algorithm that uses compositional similarity to known PFDs to identify new prion proteins is predicated on the assumption that compositional changes that reduce the biases seen in known PFDs will reduce prion propensity; thus, such algorithms assume that increasing hydrophobic content will reduce prion propensity. At the same time, hydrophobic residues have long been thought to promote amyloid formation in the context of non-Q/N-rich proteins [175], although the applicability of these results to Q/N-rich proteins is unclear. Specifically, a number of algorithms, including Waltz [4], Zyggregator [176], ZipperDB [177], and TANGO [178], have been developed that can accurately predict the aggregation propensity of non-Q/N-rich domains; each of these algorithms favors hydrophobic residues, yet none of these algorithms are able to distinguish between Q/N-rich proteins with and without prion activity, making it unclear the extent to which results from non-Q/N-rich amyloid proteins can be applied to Q/N-rich proteins.

A recent study raised further doubts about the ability of hydrophobic residues to promote prion activity. Although expanded poly-glutamine tracts show high aggregation propensity, they do not propagate efficiently as prions in yeast because they are poorly fragmented by the chaperone machinery [179]; such fragmentation is required to maintain prions over multiple generations of cell division. Alexandrov *et al*. recently showed that insertion of aromatic residues into poly-Q tracts promotes fiber fragmentation, but that non-aromatic hydrophobic residues do not exert the same positive effect [124].

There are a number of possible hypotheses that could explain why non-aromatic hydrophobic residues are so rare in yeast PFDs and fail to promote prion activity when inserted

into poly-Q tracts, yet showed high prion propensities in the screen used to develop PAPA. The simplest explanation is that the predicted prion-propensity values are either an artifact of the region tested or simply inaccurate. For example, because the prion propensity values for each amino acid were derived by random sampling, these values have large confidence intervals, so the non-aromatic hydrophobic residues may simply be less prion-prone than we predicted [172]. However, we hypothesized a more nuanced explanation. Aggregation and prion maintenance are distinct activities that appear to have distinct compositional requirements [180]. The Alexandrov experiments focused on prion maintenance. By contrast, the PAPA scores do not separate these two activities, so likely reflect some combination of the two. While non-aromatic hydrophobic residues appear unable to promote fiber fragmentation, they may still promote prion formation; in this case, aromatic hydrophobic residues may simply be favored in yeast PFDs because they can serve a dual role, promoting both prion formation and prion maintenance. To test this hypothesis, we specifically examined the effects of non-aromatic hydrophobic residues on prion formation by Sup35. We found that non-aromatic hydrophobic residues can promote prion formation to a remarkable degree. These results, combined with bioinformatics analysis of prion and non-prion Q/N-rich domains, provide insight into a number of unanswered questions about the sequence basis for prion activity.

Experimental Procedures

Strains and media. Standard yeast media and methods were used, as described previously[181], except that yeast extract-peptone-dextrose (YPD) contained 0.5% yeast extract instead of the standard 1%. In all experiments, yeast were grown at 30°C. Experiments were performed with *Saccharomyces cerevisiae* strain YER632/pJ533 (α *kar1-1 SUQ5 ade2-1 his3 leu2 trp1 ura3*

sup35::KanMx [*psi*$^-$] [*PIN*$^+$]; pJ533 expresses *SUP35* from a *URA3* plasmid as the sole copy of *SUP35* in the cell), a [*psi*$^-$] version of 780-1D/pJ533 [182].

Design of the mutants. For the hydrophobic insertions, the Excel random number function was used to select positions for insertion between amino acids 8-24 of Sup35. In each case, an equal number of isoleucines and valines were inserted. For the tyrosine deletions, the Excel random number function was likewise used to select which tyrosines should be deleted.

Cloning. CEN plasmids expressing full-length Sup35 mutants from the *SUP35* promoter were generated using homologous recombination. The mutations were inserted into the N domain of *SUP35* in two steps. For each mutant, two PCR reactions were set up. The N-terminal portion of *SUP35* was amplified with EDR302 and a mutant-specific primer, while the C-terminal portion of *SUP35* was amplified with EDR262 and a second mutant-specific primer (see Supplemental Table S1 for a complete list of primer sequences). Products of these two reactions were combined and reamplified with EDR301 and EDR262. The final PCR products were co-transformed with HindIII/BamHI-cut pJ526 [183] into yeast strain YER632/pJ533. Transformations were selected on SC-Leu, and then transferred to FOA plates to select for loss of pJ533.

To generate induction plasmids, the NM domain of each mutant was amplified by PCR using primers EDR1008 and EDR1084. EDR1084 installs a stop codon and XhoI restriction site at the end of the middle (M) domain, while EDR1008 installs a BamHI restriction site before the Sup35 start codon. PCR products were digested with BamHI and XhoI, and then inserted into BamHI/XhoI cut pKT24, a *TRP1* 2μm plasmid containing the *GAL1* promoter [183]. Ligation products were transformed into *Escherichia coli* and analyzed by DNA sequencing.

To generate vectors expressing GFP fusions, first the cassette containing the *GAL1* promoter and *ADH1* terminator was amplified from pKT24 using primers EDR1747 and EDR1748, which install SphI and EcoRI sites, respectively. This product was digested with SphI and EcoRI and then inserted into SphI/EcoRI cut YEplac112 [184] to generate plasmid pER687. Yeast-optimized GFP was then amplified from pYGFP [185] using primers EDR1898 and EDR1899, which add BamHI and SalI restriction sites, respectively, to the 5' and 3' ends of GFP. PCR products were digested with BamHI and SalI, and then inserted into BamHI/XhoI cut pER687, generating plasmid pER760. The NM domain of the Sup35 mutants were then amplified with EDR1008 and EDR1924, which add BamHI and XhoI restriction sites, respectively, to the 5' and 3' ends of the Sup35 NM. PCR products were digested with BamHI and XhoI, and then inserted into BamHI/XhoI cut pER760.

Western blot. Western blots were performed as previously described ([186]), using a monoclonal antibody against Sup35's C-terminal domain (BE4 [187], from Cocalico Biologicals, kindly made available by Susan Liebman).

[*PSI*$^+$] formation. For all prion formation assays except those for the hydrophobic rearrangement constructs, strains were transformed with either pKT24 or with a derivative of pKT24 in which the respective PFD was inserted under control of the *GAL1* promoter. Strains were grown for 3 days in galactose/raffinose dropout medium lacking tryptophan to select for pKT24 or the pKT24 derivative. It is not necessary to maintain selection for the plasmid expressing the full-length Sup35 mutant, because this plasmid expresses the only copy of *SUP35* in the cells, and *SUP35* is an essential gene. Serial 10-fold dilutions were spotted onto SC-ade medium to select for [*PSI*$^+$] cells and grown for 5 days. Although new colonies will continue to appear after 5 days, we find that these colonies tend to be unable to propagate the Ade$^+$ phenotype when removed from selection.

The hydrophobic rearrangement constructs were only tested under uninduced conditions. These strains were grown in YPAD for 2 days, and then serial 10-fold dilutions were spotted onto SC-ade medium to select for [*PSI*$^+$] cells and grown for 5 days.

Protein expression and purification The NM domain of Sup35 was recombinantly expressed on a pET-17b expression vector in BL21 CodonPlus competent cells (Agilent Technologies, CAT#230245). A one liter 2xYT culture was grown to an OD600 of 1.0. Cells were induced with 0.5 mM IPTG for four hours. Cultures were centrifuged and pellets stored at -70˚C. Protein was purified under denaturing conditions in two steps. First, cells were resuspended in lysis buffer (6M GuHCl, 0.1 M KH_2PO_4, 10 mM Tris Base, 0.05% Tween 20, pH 8) and lysed by sonication. The lysate was loaded onto a Ni-NTA sepharose column (GE Healthcare, 17-5286-01). Sup35NM was eluted with imidazole buffer (6M urea, 0.1 M KH_2PO_4, 10 mM Tris Base, 0.05% Tween 20, 0.5 M imidazole, pH 8). Second, fractions containing protein were pooled and diluted 1:4 into loading buffer (6 M urea, 50 mM MES pH 6.0). Sup35NM was loaded onto

an SP Sepharose Ion exchange column and eluted in high salt (6 M urea, 50 mM MES pH 6.0, 1 M NaCl). Fractions containing Sup35NM protein were pooled and concentrated using an Amicon Ultra centrifugal filter (Fisher, UFC901008). Protein was stored in urea at -70° C.

***In vitro* amyloid aggregation assay.** The *in vitro* assays were performed using a protocol adapted from Collins *et al*. [188]. Briefly, reactions were set up as follows: A 96-well plate (Fisher, 07-200-567) was treated with 5% casein solution for five minutes at room temperature, then rinsed with DI water and allowed to dry. Protein and thioflavin-T stock solution were diluted to a final concentration of 5 and 25 μM, respectively, in 50 mM glycine buffer, with a final reaction volume of 200 μl. Fluorescence was monitored in a Victor3 Perkin Elmer fluorescence plate reader, with excitation and emission wavelengths of 460 and 490 nm, respectively. Reactions were monitored for 48 h. Between readings, reactions were incubated without agitation for 3 minutes, and then shaken for 10 sec. The fraction aggregated was calculated by normalizing relative to the final fluorescence of the well.

Bioinformatics analysis of the yeast proteome. The complete set of systematically-named *Saccharomyces cerevisiae* open reading frames was downloaded from the Saccharomyces Genome Database (http://downloads.yeastgenome.org/sequence/S288C_reference/orf_protein/). To generate a histogram of the compositional distribution of the yeast proteome, the proteome was scanned using a 100-amino acid window size, scoring the amino acid composition of each window.

Results

Insertion of hydrophobic residues increases prion formation.

Various studies suggest that a key difference between aromatic and hydrophobic residues in the context of Q/N-rich domains is that aromatic residues facilitate the chaperone-dependent fragmentation that is required for prion maintenance [124, 189]. However, the relative effects of aromatic and hydrophobic residues on prion formation are less clear. To specifically focus on the effects of hydrophobic residues on prion formation, we took advantage of the fact that the prion formation and maintenance activities of the Sup35 PFD largely reside in separate regions of the PFD [190]. The first 40 amino acids are highly enriched in Q/N residues and are required for prion nucleation and fiber growth, while amino acids 40-114 are thought to be primarily involved in prion maintenance [180, 190-193]. To test the effect of non-aromatic hydrophobic residues on prion formation, we generated four constructs in which we inserted isoleucine or valine at random positions between residues 8-24 of Sup35, a region of the nucleation domain that is particularly important for prion activity [191]. Isoleucine and valine were chosen because they score as the most prion-promoting non-aromatic amino acids according to PAPA; leucine actually scores as slightly prion-inhibiting, likely due to its low β-sheet propensity [172]. Four *SUP35* mutants were generated (Figure 2.1A, B): two in which two hydrophobic residues were inserted into random locations in the nucleating domain of Sup35p (called +2HydA and +2HydB), and two in which six hydrophobic residues were inserted (+6HydA and +6HydB). Each mutant was cloned into a CEN plasmid under the control of the *SUP35* promoter. These plasmids were shuffled into a yeast strain that lacks an endogenous copy of *SUP35*, but carries a maintainer copy expressed from a *URA3* plasmid. After selection for loss of the maintainer plasmid, strains were tested for their propensity to convert to [*PSI*$^+$]. [*PSI*$^+$] was detected by

monitoring nonsense suppression of the *ade2-1* allele [194]. [*psi*-] *ade2-1* mutants cannot grow in the absence of adenine and form red colonies in the presence of limiting adenine due to accumulation of a pigment derived from the substrate of Ade2. However, [PSI^+] allows for occasional read-through of the *ade2-1* nonsense mutation. Thus, [PSI^+] cells can grow in the absence of adenine, and grow white in the presence of limiting adenine.

Insertion of hydrophobic residues substantially increased the frequency of Ade^+ colony formation (Figure 2.1C).

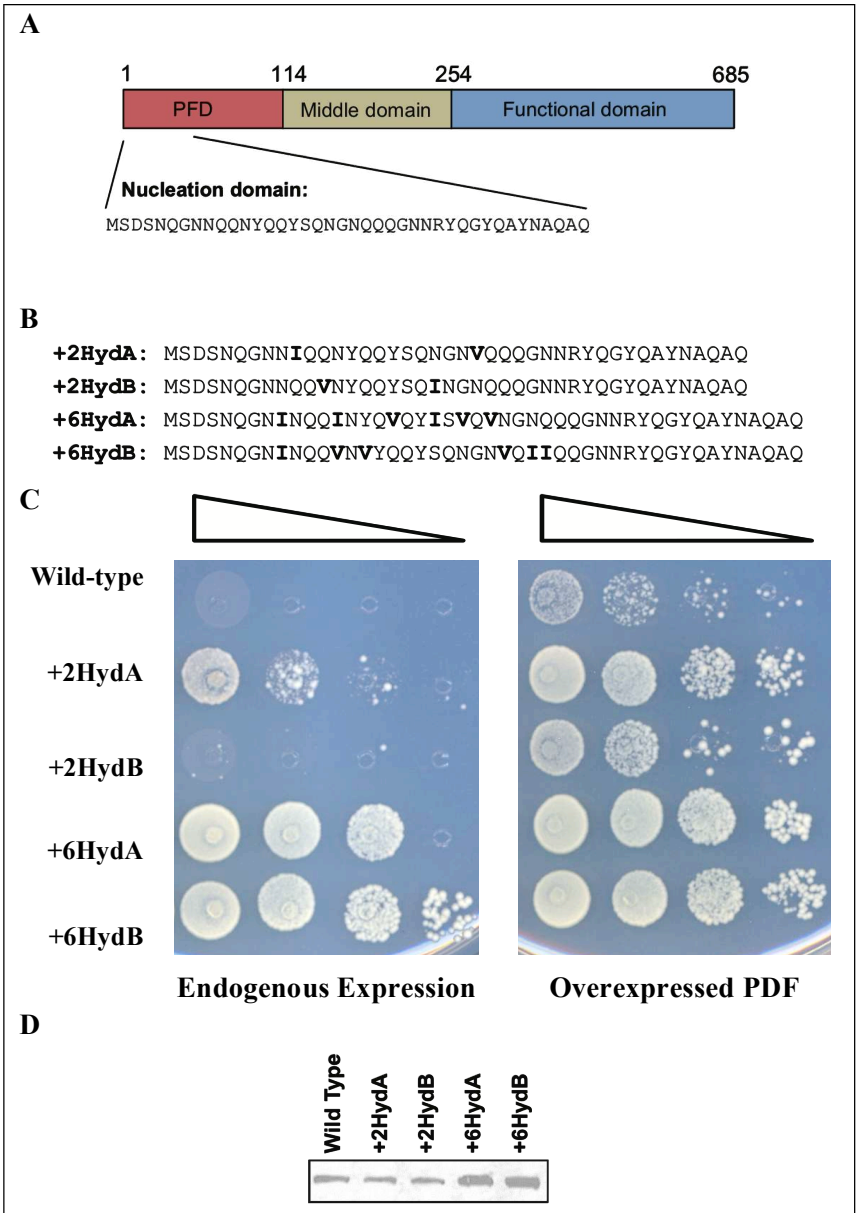


Figure 2.1. Insertion of hydrophobic residues increases prion formation.(A) Schematic of Sup35. The sequence of the nucleation domain (amino acids 1-40) is shown. (B) Sequences of the nucleation domains of each of the hydrophobic-addition constructs. Inserted hydrophobic residues are indicated in bold. For each, the remainder of the protein is the same as wild-type Sup35. (C) Prion formation by each construct. Strains expressing the indicated Sup35 mutants as the sole copy of Sup35 were transformed either with an empty vector (left) or with a plasmid expressing the matching Sup35 mutant under control of the *GAL1* promoter (right). All strains were cultured for three days in galactose/raffinose dropout medium, and then 10-fold serial dilutions were plated onto medium lacking adenine to select for [PSI^+]. (D) Western blot of wild-type and mutant Sup35.

Spontaneous wild-type prion formation is an extremely rare event, occurring in approximately one cell per million when Sup35 is expressed at endogenous levels [195]. Efficient [*PSI*$^+$]formation requires PFD overexpression, which increases the pool of soluble protein, thereby increasing the probability of the nucleation events that initiate prion formation (Figure 2.1C, right versus left panel) [50]. By contrast, the addition of six hydrophobic residues generated strains that appeared to be constantly [*PSI*$^+$], even in the absence of PFD overexpression (Figure 2.1C, left panel). This prion-promoting effect was so strong that the cells were even able to form [*PSI*$^+$] in the absence of [*PIN*$^+$], a prion required for wild-type Sup35 to form prions (Data not shown) [196]. Even just two additional hydrophobic residues caused a significant increase in frequency of Ade$^+$ colony formation, with roughly one in ten cells expressing the +2HydA construct forming Ade$^+$ colonies in the absence of PFD overexpression (Figure 2.1C, right versus left panel). This increase was not due to changes in protein levels; although the +6Hyd constructs both showed modestly higher protein levels by western blot than wild-type Sup35, protein levels for the +2Hyd constructs were similar to wild-type Sup35 (Figure 2.1D).

Despite having identical amino acid compositions, the +2HydA and +2HydB constructs showed substantial differences in frequency of prion formation. This supports the idea that while amino acid composition is the dominant factor affecting prion propensity, primary sequence also exerts an effect [105, 168, 183].

To confirm that the Ade$^+$ colonies were due to prion formation, Ade$^+$ isolates from each mutant were tested for stability and curability. Guanidine hydrochloride cures yeast prions by disrupting the activity of Hsp104 [197, 198], a chaperone protein involved in prion propagation [199-201]. Individual Ade$^+$ isolates were streaked on YPD, with and without the addition of 4

mM guanidine. Cells were then tested for loss of [*PSI*$^+$] by re-streaking onto medium containing limiting adenine. The majority of the Ade$^+$ isolates from each of the hydrophobic addition constructs were stably Ade$^+$ in the absence of guanidine, but lost the Ade$^+$ phenotype after growth on guanidine (data not shown), demonstrating that the phenotype was the result of a prion. Therefore, addition of hydrophobic residues dramatically increases the frequency of prion formation, without interfering with prion propagation.

Interestingly, some of the constructs rapidly reverted to the [*PSI*$^+$] after curing. The most extreme was the +6A construct. It formed predominantly weak prions, as indicated by a pink phenotype. Although these cells were fully red on guanidine medium (data not shown), upon restreaking onto non-selective medium, they rapidly converted to a mixture of red, white, pink, and sectored colonies.

To ensure that the observed differences in prion formation were due to changes in prion propensity, rather than an artifact such as mislocalization, differences in toxicity, or alteration of a prion-modifying protein-protein interaction, the mutants were purified

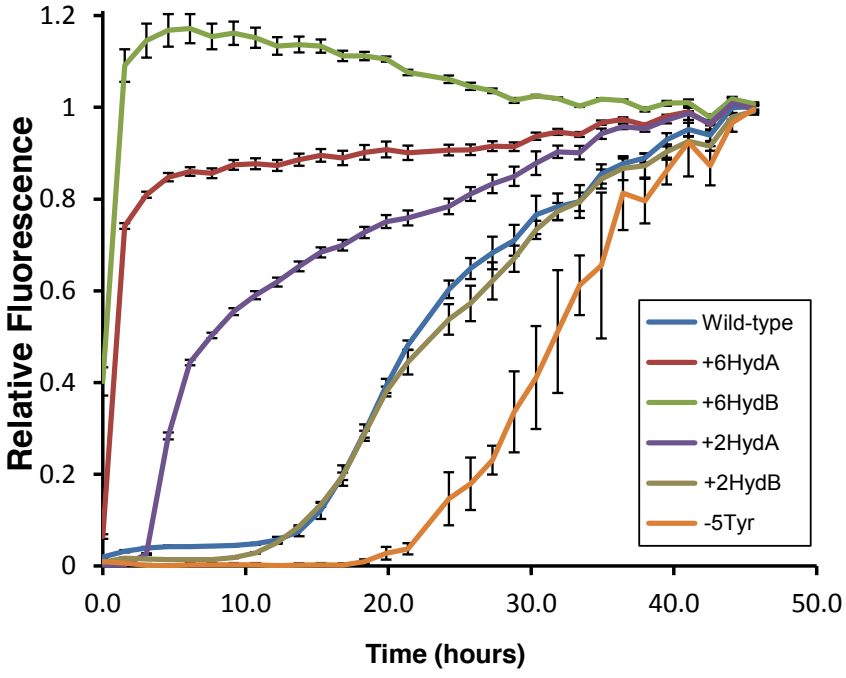


Figure 2.2 *In vitro* amyloid aggregation of the mutant prion forming domains. Aggregation of purified PFDs was monitored using thioflavin T. Reactions were incubated with intermittent shaking for 48 h. Fluorescent readings were taken approximately every 90 min. Error bars represent the standard deviations of three samples.

and assayed for amyloid formation *in vitro* (Figure 2.2). Amyloid aggregation was monitored using Thioflavin T, a dye

that forms fluorescent complexes with amyloid fibrils, but not with soluble proteins or amorphous aggregates [202]. In each case, the rate of aggregation *in vitro* correlated well with prion formation *in vivo* (Figure 2.1C). As expected, wild-type Sup35 had a lag phase lasting approximately 9 hours before aggregation and increased in a roughly sigmoid fashion. Remarkably, +6HydA and +6HydB each showed no detectable lag phase and plateaued within three hours.

The effect of primary sequence on prion formation

Both +2HydA and +2HydB carry an extra isoleucine and valine. The observed prion formation differences between these compositionally identical constructs demonstrate that small changes in primary sequence can exert substantial effects on prion formation. Therefore, these constructs provide a useful system to explore the basis for such primary sequence effects.

However, systematically repositioning the isoleucine and valine did not reveal any clear trend (Figure 2.3A,B). The constructs showed substantial differences in both the number of Ade^+ colonies observed and the fraction of these colonies that propagated as stable, guanidine-curable prions (Figure 2.3B). Western blot showed only small expression differences among the mutants, and neither the frequency of Ade^+ colony formation nor the stability of the Ade^+ phenotype consistently correlated with expression levels (Figure 2.3C). Unexpectedly, the +2HydD mutant showed two bands: a predominant band at the expected size, and a minor band running at a higher molecular weight. This raises the possibility that a subset of the +2HydD protein pool could be undergoing modification, suggesting that prion formation levels for this mutant should be interpreted with caution.

These large differences in prion activity are not predicted by any of the commonly-used aggregation prediction algorithms. Not surprisingly, composition-based algorithms such as PAPA or Zyggregator were not effective at distinguishing among these constructs. However, while the prion propensity of Q/N-rich domains is predominantly determined by amino acid composition, a variety of evidence suggests that short sequence motifs may play a critical role in nucleating prion formation by Sup35 [203-205]. Thus, the mutations may affect prion activity by creating or disrupting amyloid-promoting primary sequence motifs. The Serrano group has used both computational and experimental techniques to determine a consensus hexameric sequence that promotes

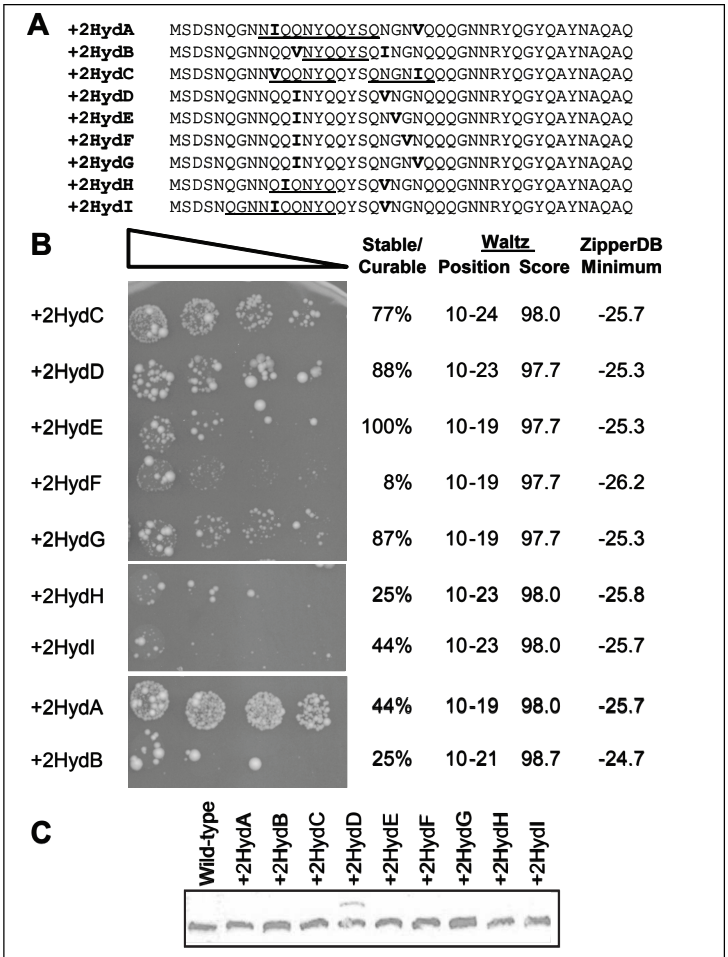


Figure 2.3. Effects of primary sequence on prion formation. (A) Amino acid sequences of constructs in which two additional hydrophobic residues were added at various positions within the Sup35 nucleation domain. For each, the remainder of the protein is the same as wild-type Sup35. Amyloid stretches, as predicted by Lopez de la Paz and Serrano [3], are underlined. The inserted hydrophobic residues are indicated in bold. (B) Prion formation by each of the constructs. Strains expressing the indicated Sup35 mutants as the sole copy of Sup35 were grown in YPAD medium for two days, and then 10-fold serial dilutions were plated onto medium lacking adenine to select for [*PSI*$^+$]. For each construct, the position and scores of amyloid stretches predicted by Waltz [4], as well as the minimum ZipperDB score [5], are indicated. A minimum of 8 Ade$^+$ colonies (and more when possible) were tested for stability and curability. (C) Western blot of expression levels of wild-type and mutant Sup35s.

amyloid formation [3, 206]. Interestingly, the only such stretch in the nucleation domain of Sup35 overlaps with the region mutated in these constructs (Figure 2.3A). However, there did not appear to be any correlation between the presence of such stretches and prion activity (Figure 2.3A,B).

Other prediction algorithms were no more effective. A recent, more comprehensive study has expanded the definition of the hexameric amyloid stretch [4]. The prediction algorithm Waltz utilizes this broader definition and provides quantitative scores for different stretches. However, no correlation was seen between Waltz scores and Ade$^+$ colony formation. All of the proteins had Waltz-positive segments; although there were differences

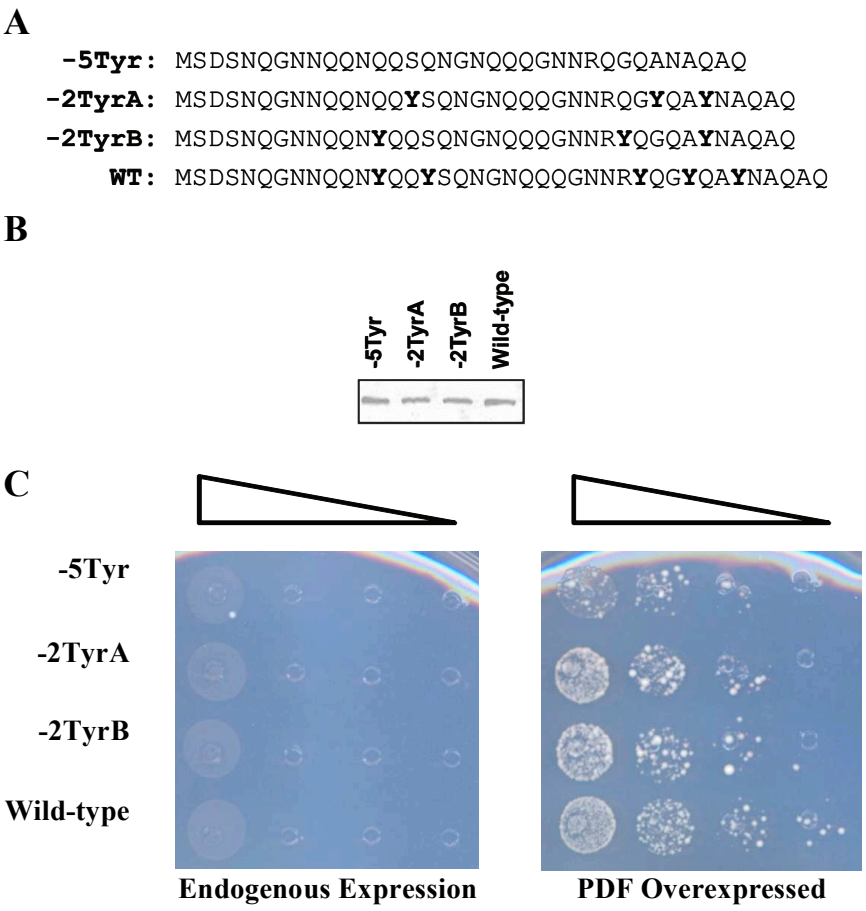


Figure 2.4 Deletion of tyrosine residues reduces prion formation. (A) Amino acid sequences of constructs in which tyrosines were deleted from various positions within the Sup35 nucleation domain. Tyrosines are indicated in bold. (B) Western blot of expression levels of wild-type and mutant Sup35s. (C) Prion formation by each construct. Strains expressing the indicated Sup35 mutants as the sole copy of Sup35 were transformed either with an empty vector (left) or with a plasmid expressing the matching Sup35 mutant under control of the *GAL1* promoter (right). All strains were cultured for three days in galactose/raffinose dropout medium, and then 10-fold serial dilutions were plated onto medium lacking adenine to select for [*PSI*$^+$].

in the length of these segments, there was no clear correlation between the length or score of the predicted amyloid stretch and observed prion activity. The same was true using the "High Specificity" setting, which is intended to reduce false positives; again, all constructs had Waltz-positive segments overlapping with the mutated region, and there was no correlation between the length of the predicted amyloid stretch and observed prion activity (data not shown).

Similar results were seen for ZipperDB, another algorithm that looks for 6-amino-acid aggregation-prone segments. ZipperDB is a structure-based prediction method. Sequences are threaded into a known NNQQNY amyloid-forming hexapeptide crystal structure and the energetic fit is determined [5, 177]. Segments with a free energy below -23 kcal/mol are considered to have high fibrillation propensity; insertion of a single such sequence into a loop region of RNase A was sufficient to cause amyloid formation [207]. All of the +2Hyd constructs had segments well below -23 kcal/mol that overlapped with the mutated region; however, there was no correlation between the predicted free energy and the observed frequency of prion activity (Figure 2.3B). In short, while it is clear that primary sequence effects do exist, none of the commonly used amyloid prediction algorithms successfully predict these effects.

Deletion of tyrosine residues reduces prion formation and aggregation

The Sup35 nucleation domain shows a striking under-representation of highly hydrophobic residues, completely lacking F, I, L, M, W or V; however, it does contain five tyrosines. Three mutants were generated in which either two (-2TyrA and -2TyrB) or five of these tyrosines (-5Tyr) were eliminated from the nucleation domain (Figure 2.4A). Each was expressed at levels comparable to wild-type Sup35 (Figure 2.4B). None of these mutants showed detectable Ade^+ colony formation when expressed at endogenous levels (Figure 2.4C).

However, because even wild-type Sup35 only rarely forms prions without Sup35 overexpression, it remained possible that the tyrosine deletion mutants were simply forming prions at a frequency below the threshold of detection. Indeed, transient over-expression of the corresponding PFD increased Ade^+ colony formation by each of the strains (Figure 2.4C), suggesting that each of the mutants is capable of prion formation. However, the -5Tyr construct showed substantially reduced frequencies of Ade+ colony formation (Figure 2.4C). When tested for stability and curability, all Ade^+ colonies isolated from the tyrosine deletion mutants were red after growth both with and without guanidine, indicating that the Ade^+ phenotype is unstable (data not shown).

Additionally, PFD-GFP fusions showed substantially reduced foci formation. For wild-type Sup35, over-expression of PFD-GFP fusions results in the formation of fluorescent foci (Figure 2.5A). Likewise, large foci were consistently observed for each of the hydrophobic addition constructs (Figure 2.5B). However, no foci were observed in cells expressing -2TyrA or -5Tyr (Figure 2.5C), while foci were observed in only a subset of the cells expressing -2TyrB (Figure

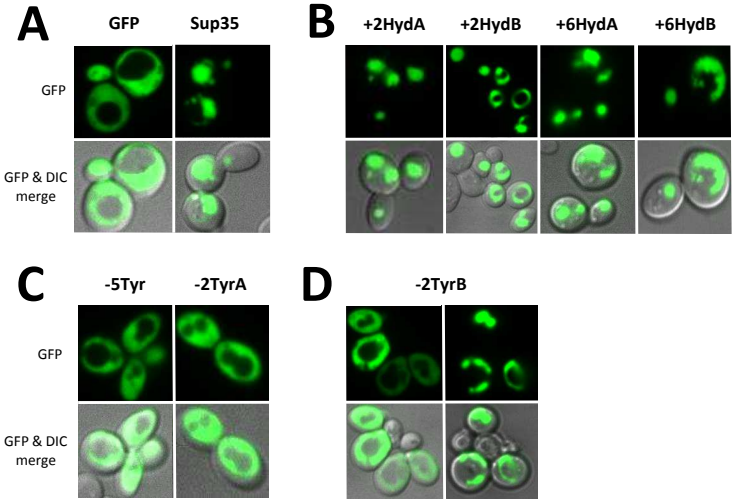


Figure 2.5 Tyrosine and hydrophobic residues promote foci formation. (A) The Sup35 PFD promotes formation of fluorescent foci. GFP or the NM domain from wild-type Sup35 fused to GFP were expressed under control of the *GAL1* promoter. Cells were grown in galactose/raffinose dropout medium for 24 h, and then visualized by confocal microscopy. (B) The hydrophobic insertion constructs each support formation of fluorescent foci. Conditions were as described in (A). (C) The -5Try and -2TyrA constructs fail to form fluorescent foci. (D) The -2TyrB construct forms foci in a fraction of cells.

2.5D). Therefore, the tyrosine deletion mutants show substantially reduced *in vivo* aggregation, and appear completely unable to form stable [*PSI*$^{+}$] prions.

Some prion variants formed by wild-type Sup35 are either deleterious or lethal to yeast cells [143]. Therefore, the reduced prion formation and aggregation by the -5Tyr mutant could theoretically result from an artifact such as an increase in prion toxicity. However, the -5Tyr construct also showed substantially reduced aggregation kinetics *in vitro* (Figure 2.2), suggesting that tyrosine deletion directly affects aggregation propensity.

The effect of aromatic residues on prion formation.

Although there are very few highly hydrophobic residues in yeast PFDs, tyrosines are over-represented among a subset of yeast PFDs [166]. We directly compared the ability of hydrophobic and aromatic residues to drive prion formation by replacing the five native tyrosines in the Sup35 nucleation domain with

leucines, isoleucines, or valines. All three constructs were able to form Ade^{+} colonies, albeit at different frequencies, and all three showed more Ade^{+} colony formation with PFD over-expression than without, consistent with the Ade^{+} colonies resulting from prion formation (Figure 2.6A). However, there were substantial differences both in the frequency of Ade^{+} colony formation (Figure 2.6A) and the fraction of these colonies that propagated as stable, curable prions (Figure 2.6B-E). The valine substitution construct showed the highest frequency of Ade^{+} colony formation (Figure 2.6A); however, the Ade^{+} colonies were consistently unstable, rapidly losing the Ade^{+} phenotype upon growth on non-selective medium (Figure 2.6D). While the construct with isoleucine substitutions showed less Ade^{+} colony formation, the majority of the Ade^{+} colonies were stable and curable (Figure 2.6E). The construct with leucine substitutions only formed small Ade^{+} colonies, and none of the Ade^{+} isolates were able to maintain the Ade^{+}

phenotype upon non-selective growth (Figure 2.6C). This result is consistent with the relatively low prion propensity of leucine compared to the other hydrophobic residues [172], although it is possible that this failure to form stable prions is a result of a less direct effect, such as increased toxicity of prions formed by this mutant. Therefore, while the identity of the hydrophobic residues within the nucleation domain affects both prion formation and prion stability, there is no strict requirement for aromatic residues within the prion-nucleating domain of Sup35.

Compositional biases in glutamine/asparagine rich domains.

The substantial effects of insertion or deletion of hydrophobic and aromatic residues in Sup35 highlight the narrow prion-propensity window required for a protein to act as a prion. According to PAPA, there are six strongly prion-promoting amino acids: F, I, V, Y, M and W. These amino acids have similar prion propensity scores, and are all predicted to be substantially more prion-prone than any other amino acid [172]. The 114-amino-acid Sup35 PFD contains 23 of these prion-promoting amino acids, representing 20.2% of the PFD; increasing this number to 24.2% in the +6Hyd constructs almost completely eliminated the soluble, functional state. It is likely that the exact number of prion-promoting residues required for prion activity is somewhat context-dependent; however, based on this dramatic effect of hydrophobic insertions, we hypothesized that it would be unlikely that any Q/N-rich regions in yeast would contain substantially more prion-promoting residues than Sup35. Indeed, this appears to be true.

In order to better evaluate regions of high compositional bias we developed a script, dubbed GARRF (Glutamine Asparagine Rich Region Finder) that scans through genomic data and returns domains with Q/N content above a set threshold. There are a couple of bioinformatics data sets in the literature that sort out proteins with high Q/N content, but developing our own algorithm provided a couple of advantages. GARRF scans through a protein

one residue at a time recording the Q/N content within a 10-residue window. When it finds 10 residues with a user-defined minimum Q/N content, it jumps to the adjacent 10-residue window and measures its Q/N content. It keeps scanning through a protein in 10-residue blocks until it reaches a block in which Q/N content falls below the user-defined minimum. The resulting contiguous domain is recorded in a data file for future analysis (see Chapter 3 for a detailed description of the algorithm). Thus, GARRF is capable of identifying multiple Q/N-rich domains inside of a single protein, something no other published algorithm can do.

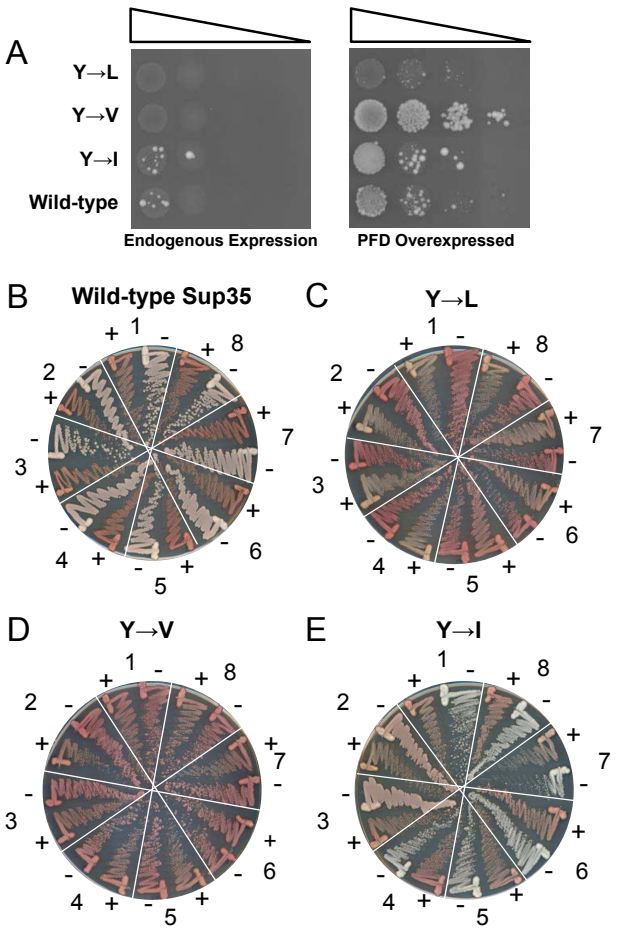


Figure 2.6 Aromatic residues are not required in the Sup35 nucleation domain.
(A) The five tyrosines in the Sup35 nucleation domain (amino acids 1-40) were replaced with either leucines, isoleucines or valines. Strains were transformed either with an empty vector (left) or with a plasmid expressing the matching Sup35 mutant under control of the *GAL1* promoter (right). All strains were cultured for three days in galactose/raffinose dropout medium, and then 10-fold serial dilutions were plated onto medium lacking adenine to select for [*PSI*$^+$]. (B-E) Stability and curability of the Ade$^+$ phenotype in cells expressing wild-type Sup35 (B), or Sup35 in which the five tyrosines in the nucleation domain were replaced with leucine (C), valine (D) or isoleucine (E). For each mutant, eight individual Ade$^+$ isolates were grown on YPD (-) and YPD plus 4 mM guanidine HCl (+). Cells were then restreaked onto YPD to test for loss of the Ade$^+$ phenotype.

If our analysis is restricted to proteins with greater than or equal to 30% Q/N content and a minimum length of 40 residues,

GARRF identifies 329 regions in the yeast proteome with strong Q/N bias. There is substantial diversity in the function and composition of these regions;
they are as long as 160 residues, and range from 30 to 73% Q/N content. Nevertheless, only a few of these 170 Q/N-rich domains has more than the 24.2% F, I, V, Y, M and W that is found in the +6Hyd constructs (Figure 2.7A). By contrast, when the FIVYM content of every protein in the yeast proteome is calculated, over half of all protein fragments have more than the 24.2% F, I, V, Y, M and W. Moreover, although F, I, V, Y, M and W constitute 23.0% of the yeast proteome, the Q/N-rich regions contain on average only 12.6% of these residues.

There are also subtle differences in the frequencies of strongly prion-promoting residues between the prion-forming and non-prion Q/N-rich domains. We analyzed 21 proteins with clear prion activity. Eight of these have been proven to act as prions, while an additional fourteen were shown by Alberti *et al.* to have prion-like activity in four independent assays [105] The non-prion Q/N-rich sequences show both lower average frequencies of strongly prion-promoting residues (Figure 2.7C) and a broader range (Figure 2.7B). Additionally, there appear to be differences in which strongly prion-promoting residues are found in the prion versus non-prion sequences. While the two sets have similar numbers of non-aromatic prion-promoting residues (I, V and M), the prion sequences have substantially more aromatic residues (Figure 2.7C).

The substantial bias against strongly prion-promoting residues in non-prion Q/N-rich domains could simply be a result of high Q/N content. Because these domains average about 45% Q/N residues, the high Q/N content may simply crowd out other residues. However, when the frequency of strongly prion-promoting residues is calculated as a percentage of the total number of non-Q/N residues, the prion-promoting amino acids are still slightly under-represented in non-prion Q/N-rich domains. In the yeast genome, F, I, V, Y, M and W constitute

25.7% of the non-Q/N residues (Figure 2.7D). This is similar to their average frequency in Q/N-rich PFDs; by contrast, the average among non-prion Q/N-rich domains is 20.4%. Thus, even when positions occupied by Q/N residues are excluded from the analysis, strongly prion-promoting amino acids are still slightly under-represented in non-prion Q/N-rich domains.

Finally, there are many ways that the compositional analysis of Q/N-rich domains could have been done, each of which would have unique strengths and weaknesses. Harrison and Gerstein developed an algorithm that identifies regions with statistically unlikely Q/N concentration within a genome. This data set is useful, as it contains a large collection of protein regions with strong Q/N-bias (170 domains in the *Saccharomyces cerevisiae* genome). Nevertheless, it has the disadvantage that because the algorithm uses a statistical bias for Q/N residues, it includes some very large regions that are only modestly enriched for Q/N, and it excludes some shorter regions that have relatively high Q/N content. Michelitsch and Weissman developed another search algorithm called DIANA. DIANA identified every yeast protein that contains an 80-residue window with at least 30 Q/Ns; then, within these proteins it identified the most Q/N-rich 80-amino acid segment. While this method is effective for identifying prion candidates, it was not as ideal for our purposes, as it is blind to multiple Q/N-rich domains within a single protein. Additionally, because the window size is fixed at 80 amino acids, in some cases only a portion of the 80-amino acid segment is Q/N-rich. Despite these disadvantages, the same basic trends were seen in these data sets as with the GARRF set.

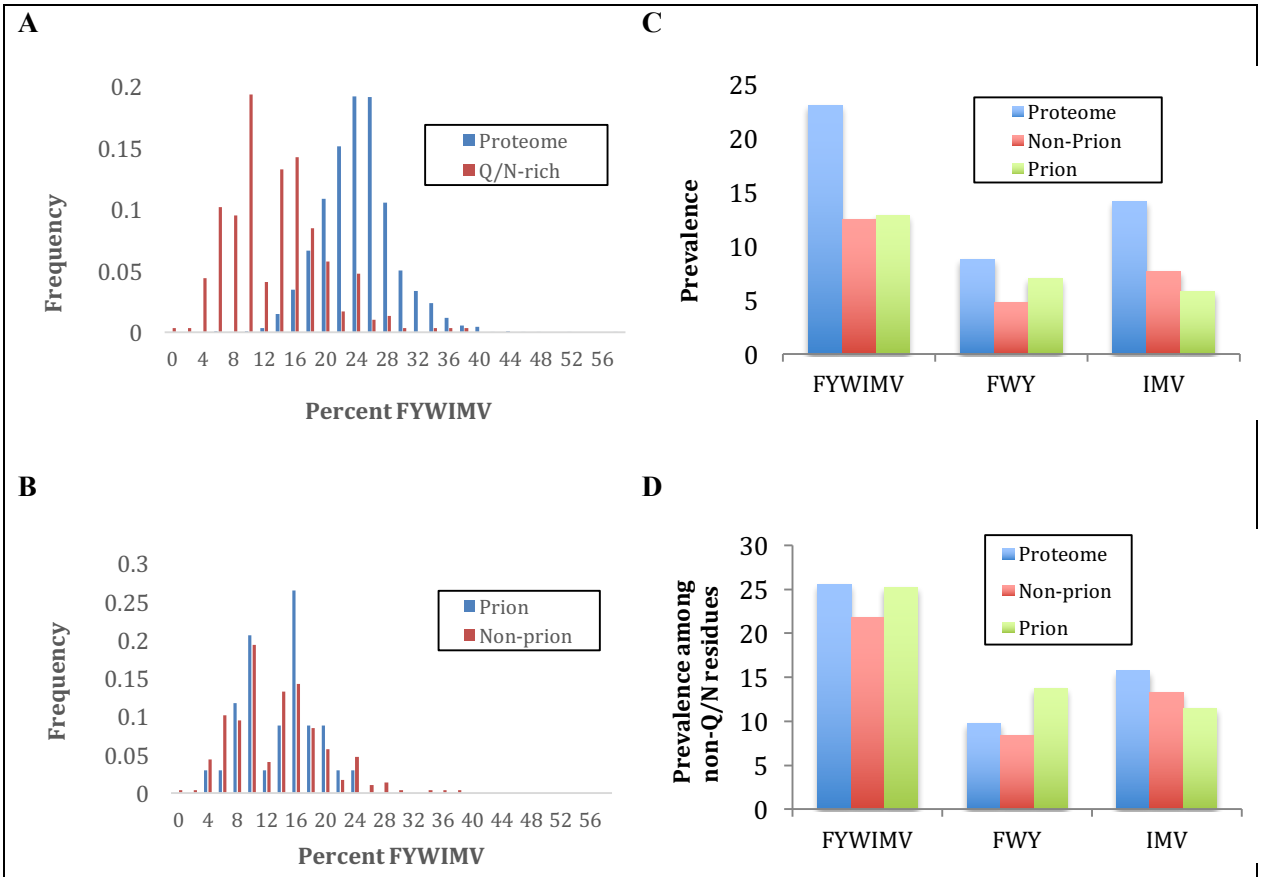


Figure 2.7 Amino acid composition of prion and non-prion Q/N-rich domains.
(A) Histogram of the prevalence of strongly prion-promoting residues (FYWIMV) among Q/N-rich proteins (orange bars) and among peptide fragments from the yeast proteome (blue bars). For the Q/N-rich proteins, each of the regions of the yeast proteome identified by GARRF as having high Q/N-bias were scored for the fraction of strongly prion-promoting amino acids. For the proteomic data, the yeast proteome was scanned and each full-length protein was scored for the fraction of strongly prion-promoting amino acids. (B) Histogram of the prevalence of strongly prion-promoting amino acids among yeast prion and non-prion Q/N-rich domains. The blue bars include Q/N-rich regions (as identified by GARRF) from yeast proteins shown to act as prions, as well as from proteins containing domains shown by Alberti *et al.* to have prion-like activity in four independent assays [105]. Orange bars represent all other yeast Q/N-rich regions identified by GARRF. (C) Amino acid prevalence in Q/N-rich domains. Blue bars represent the prevalence of different groups of amino acids in the yeast proteome. Grey bars represent the average frequency of these amino acids among Q/N-rich regions from both proteins shown to act as prions and proteins containing domains shown by Alberti *et al.* to have prion-like activity in four independent assays. Orange bars represent the average frequency of these amino acids among all other yeast Q/N-rich domains identified GARRF. (D) The prevalence of different groups of amino acids, plotted as a fraction of non-Q/N residues.

Discussion

We previously scored the prion propensity of each amino acid in the context of a Q/N-rich PFD [172], and were surprised to find that there was little correlation between the amino acids that most strongly support prion formation and those that are actually found in yeast PFDs. Here, we provide an explanation for this apparent contradiction.

We first confirmed that non-aromatic hydrophobic residues do strongly promote prion formation. We found that both aromatic residues and non-aromatic hydrophobic residues (with the exception of leucine) all promote prion nucleation, albeit to varying degrees, demonstrating that our previous prion-propensity estimates were not an artifact of the region tested or a product of sampling error. This effect was even stronger than we anticipated, and suggests that prion formation can easily be controlled by modifying the number and position of hydrophobic residues. However, the question remained, if these residues promote prion formation, why are they so rare in actual PFDs?

Combined with our experimental data, our bioinformatics analysis suggests an answer to this question. Strongly prion-promoting residues (F, W, Y, I, V and M) are under-represented among both prion and non-prion Q/N-rich domains (Figure 2.7), most likely because too many of these residues would make proteins excessively aggregation-prone. A variety of evidence indicates that aromatic residues facilitate prion maintenance [124, 189]. This requirement for aromatic residues, coupled with a limit on the number of strongly prion-promoting residues tolerated in Q/N-rich domains, likely leads to the exclusion of non-aromatic residues from yeast PFDs. It should be noted that one study suggests that another difference between aromatic and non-aromatic hydrophobics is that aromatic residues, but not non-aromatic hydrophobic residues, can make contacts that facilitate the early oligomerization steps in prion formation [208].

However, in that study, leucine was used as the non-aromatic hydrophobic residue; leucine is uniquely non-prion-prone among the hydrophobic residues [172], presumably due to its low β-sheet propensity [209], so additional studies will be needed to determine whether this result applies to all hydrophobic residues. Regardless, our results strongly argue that hydrophobic residues are rare not because they inhibit prion formation, but because aromatic residues are equally able to support prion formation, and can also contribute to other steps in prion activity.

Indeed, while it has been well-documented that non-aromatic hydrophobic residues are under-represented in yeast PFDs [166, 210], the fact that these residues are almost equally rare among non-prion Q/N-rich domains is often ignored. This highlights a key point: the sequence features that most clearly distinguish Q/N-rich PFDs from the entire proteome may not be the same features that most effectively distinguish between prion and non-prion Q/N-rich domains. This distinction likely explains why algorithms designed to identify new prions based on compositional similarity to existing prions are very effective at identifying prion candidates, yet are far less effective at ranking the top candidates [172]. Unfortunately, this distinction continues to be missed. For example, a recent paper by Espinosa Angarica *et al.* argued that because C, W and E are rare among yeast PFDs, these residues must make an "unfavorable contribution" to prion activity [210]; however, this analysis ignores the fact that while W is rare among yeast PFDs, it is even more rare among non-prion Q/N-rich domains.

One key caveat with these experiments is that different regions of PFDs may have different sequence requirements, based on their respective roles in prion activity. For Sup35, the nucleation domain and remainder of the prion domain (termed the oligopeptide repeat domain, due to the presence of a series of imperfect peptide repeats) have both distinct roles in prion activity [190] and distinct compositional requirements [180]. By focusing on the nucleation

domain, we were able to specifically isolate the effects of hydrophobic residues on prion formation. In the nucleation domain, non-aromatic hydrophobic residues and aromatic residues seem at least partially interchangeable. However, we have separately begun to systematically examine the distinct sequence requirements for prion formation versus maintenance, and have found that aromatic residues appear to play a more essential role within the ORD (unpublished data), consistent with their proposed functions in prion maintenance [124, 189].

Our results explain a number of other conundrums in the prion field. There has been substantial debate about the role of short sequence motifs in yeast prion formation. For example, a variety of evidence suggests that a short segment of the Sup35 PFD spanning amino acids 8-24 acts as a key nucleating site for prion formation, and point mutations in this region can prevent addition to prion aggregates and can substantially affect efficiency of cross-species transmission [203, 205]. However, much larger fragments are required for prion activity [190]; the shortest region from any prion protein that has be shown to support prion activity is 37 amino acids from Swi1 [211]. Furthermore, the fact that PFDs can be scrambled without blocking prion formation seems to argue against the importance of short sequence motifs. Our data provide a simple explanation for this apparent contradiction. If yeast PFDs contain relatively few strongly prion-promoting amino acids, then wherever these amino acids are located will naturally act as potential nucleating sites. Indeed, residues 8-24 of Sup35 contain two strongly prion-promoting amino acids, and no strongly inhibiting amino acids (charged residues or prolines). In fact, the longest segment in the Sup35 PFD without a prion-inhibiting amino acid spans residues 4-27. Thus, the key role of this segment in prion nucleation may be explainable solely based on composition.

Interestingly, while composition is the dominant factor in determining prion activity, our data clearly demonstrate that primary sequence can exert a substantial effect on both the frequency of prion formation and the stability of the prion phenotype. The basis for this effect is unclear. Composition-based algorithms such as PAPA clearly do not predict such a strong effect of primary sequence. However, even algorithms designed to detect primary-sequence motifs appear to be no more effective (Figure 2.3B). While this set of mutants is likely too small to extract the exact relationship between primary sequence and prion propensity, this data set could provide a useful tool for testing future primary-sequence-based algorithms. However, it is important to note that the observed differences may not be due solely to differences in prion propensity. Because some prion variants can be deleterious or lethal [143], a mutation that shifts the distribution of variants formed by the protein to more toxic variants (or increases the toxicity of common variants) could give the appearance of reducing prion propensity. Therefore, more detailed studies will be needed to untangle the basis for this primary sequence effect

Special Recognition:

We thank Olve Peersen for help with the microplate reader and Santiago Di Pietro for assistance with microscopy. The microscope used in this work is supported in part by the Microscope Imaging Network core infrastructure grant from Colorado State University.

BIBLIOGRAPHY

1. Wadsworth, J.D. and J. Collinge, *Update on human prion disease.* Biochim Biophys Acta, 2007. **1772**(6): p. 598-609.
2. Coustou, V., et al., *The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog.* Proc. Natl. Acad. Sci. USA, 1997. **94**(18): p. 9773-8.
3. Maclea, K.S. and E.D. Ross, *Strategies for identifying new prions in yeast.* Prion, 2011. **5**(4): p. 263-268.
4. Li, Y.R., et al., *Stress granules as crucibles of ALS pathogenesis.* J Cell Biol, 2013. **201**(3): p. 361-72.
5. Da Cruz, S. and D.W. Cleveland, *Understanding the role of TDP-43 and FUS/TLS in ALS and beyond.* Curr Opin Neurobiol, 2011. **21**(6): p. 904-19.
6. Geser, F., et al., *Amyotrophic lateral sclerosis, frontotemporal dementia and beyond: the TDP-43 diseases.* J Neurol, 2009. **256**(8): p. 1205-14.
7. Weihl, C.C., et al., *TDP-43 accumulation in inclusion body myopathy muscle suggests a common pathogenic mechanism with frontotemporal dementia.* J Neurol Neurosurg Psychiatry, 2008. **79**(10): p. 1186-9.
8. Couthouis, J., et al., *Evaluating the role of the FUS/TLS-related gene EWSR1 in amyotrophic lateral sclerosis.* Hum Mol Genet, 2012: p. Epub ahead of print.
9. Couthouis, J., et al., *A yeast functional screen predicts new candidate ALS disease genes.* Proc Natl Acad Sci U S A, 2011. **108**: p. 20881-20890.
10. Neumann, M., et al., *FET proteins TAF15 and EWS are selective markers that distinguish FTLD with FUS pathology from amyotrophic lateral sclerosis with FUS mutations.* Brain, 2011. **134**(Pt 9): p. 2595-609.
11. Kim, H.J., et al., *Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS.* Nature, 2013. **495**(7442): p. 467-73.
12. Klar, J., et al., *Welander distal myopathy caused by an ancient founder mutation in TIA1 associated with perturbed splicing.* Hum Mutat, 2013. **34**(4): p. 572-7.
13. Bradley, M.E. and S.W. Liebman, *The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI+] prions.* Mol. Microbiol., 2004. **51**(6): p. 1649-1659.
14. Ter-Avanesyan, M.D., et al., *The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast Saccharomyces cerevisiae.* Genetics, 1994. **137**(3): p. 671-6.
15. Ter-Avanesyan, M.D., et al., *Deletion analysis of the SUP35 gene of the yeast Saccharomyces cerevisiae reveals two non-overlapping functional regions in the encoded protein.* Mol. Microbiol., 1993. **7**(5): p. 683-92.
16. Harrison, P.M. and M. Gerstein, *A method to assess compositional bias in biological sequences and its application to prion-like glutamine/asparagine-rich domains in eukaryotic proteomes.* Genome Biol, 2003. **4**(6): p. R40.
17. Liu, J.J., N. Sondheimer, and S.L. Lindquist, *Changes in the middle region of Sup35 profoundly alter the nature of epigenetic inheritance for the yeast prion [PSI+].* Proc. Natl. Acad. Sci. USA, 2002. **99 Suppl 4**: p. 16446-53.

18. Ross, E.D., U. Baxa, and R.B. Wickner, *Scrambled Prion Domains Form Prions and Amyloid.* Mol. Cell. Biol., 2004. **24**(16): p. 7206-7213.
19. Alberti, S., et al., *A systematic survey identifies prions and illuminates sequence features of prionogenic proteins.* Cell, 2009. **137**(1): p. 146-58.
20. Michelitsch, M.D. and J.S. Weissman, *A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions.* Proc. Natl. Acad. Sci. USA, 2000. **97**(22): p. 11910-5.
21. Sondheimer, N. and S. Lindquist, *Rnq1: an epigenetic modifier of protein function in yeast.* Mol. Cell, 2000. **5**(1): p. 163-72.
22. Halfmann, R., et al., *Prion formation by a yeast GLFG nucleoporin.* Prion, 2012. **6**(4).
23. Ross, E.D. and J.A. Toombs, *The effects of amino acid composition on yeast prion formation and prion domain interactions.* Prion, 2010. **4**(2): p. 60-5.
24. Toombs, J.A., B.R. McCarty, and E.D. Ross, *Compositional determinants of prion formation in yeast.* Mol. Cell. Biol., 2010. **30**(1): p. 319-332.
25. Ross, E.D., et al., *A bioinformatics method for identifying Q/N-rich prion-like domains in proteins.* Methods Mol Biol, 2013. **1017**: p. 219-28.
26. Toombs, J.A., et al., *De novo design of synthetic prion domains.* Proc Natl Acad Sci U S A, 2012. **109**(17): p. 6519-6524.
27. Chiti, F., et al., *Rationalization of the effects of mutations on peptide and protein aggregation rates.* Nature, 2003. **424**(6950): p. 805-8.
28. Maurer-Stroh, S., et al., *Exploring the sequence determinants of amyloid structure using position-specific scoring matrices.* Nature Methods, 2010. **7**(3): p. 237-42.
29. Tartaglia, G.G., et al., *Prediction of aggregation-prone regions in structured proteins.* J Mol Biol, 2008. **380**(2): p. 425-36.
30. Goldschmidt, L., et al., *Identifying the amylome, proteins capable of forming amyloid-like fibrils.* Proc. Natl. Acad. Sci. USA 2010. **107**(8): p. 3487-3492.
31. Fernandez-Escamilla, A.M., et al., *Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins.* Nat. Biotechnol., 2004. **22**(10): p. 1302-6.
32. Salnikova, A.B., et al., *Nonsense suppression in yeast cells overproducing Sup35 (eRF3) is caused by its non-heritable amyloids.* J Biol Chem, 2005. **280**(10): p. 8808-12.
33. Alexandrov, A.I., et al., *The effects of amino acid composition of glutamine-rich domains on amyloid formation and fragmentation.* PLoS ONE, 2012. **7**(10): p. e46458.
34. Toombs, J.A., et al., *[PSI+] maintenance is dependent on the composition, not primary sequence, of the oligopeptide repeat domain. PLoS One*, 2011. **6**(7): p. e21953.
35. Sherman, F., *Getting started with yeast.* Methods Enzymol., 1991. **194**: p. 3-21.
36. Song, Y., et al., *Role for Hsp70 chaperone in Saccharomyces cerevisiae prion seed replication.* Eukaryot. Cell, 2005. **4**(2): p. 289-97.
37. Ross, E.D., et al., *Primary sequence independence for prion formation.* Proc. Natl. Acad. Sci. USA, 2005. **102**(36): p. 12825-12830.
38. Gietz, R.D. and A. Sugino, *New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites.* Gene, 1988. **74**(2): p. 527-34.
39. Cormack, B.P., et al., *Yeast-enhanced green fluorescent protein (yEGFP)a reporter of gene expression in Candida albicans.* Microbiology, 1997. **143**(Pt 2): p. 303-11.

40. Ross, C.D., et al., *A promiscuous prion: Efficient induction of [URE3] prion formation by heterologous prion domains. Genetics*, 2009. **183**(3): p. 929-40.
41. Bagriantsev, S.N., V.V. Kushnirov, and S.W. Liebman, *Analysis of amyloid aggregates using agarose gel electrophoresis.* Methods Enzymol, 2006. **412**: p. 33-48.
42. Collins, S.R., et al., *Mechanism of Prion Propagation: Amyloid Growth Occurs by Monomer Addition.* PLoS Biology, 2004. **2**(10): p. e321.
43. Alexandrov, I.M., et al., *Appearance and propagation of polyglutamine-based amyloids in yeast: tyrosine residues enable polymer fragmentation.* J Biol Chem, 2008. **283**(22): p. 15185-92.
44. Osherovich, L.Z., et al., *Dissection and design of yeast prions.* PLoS Biol., 2004. **2**(4): p. E86.
45. DePace, A.H., et al., *A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion.* Cell, 1998. **93**(7): p. 1241-52.
46. Parham, S.N., C.G. Resende, and M.F. Tuite, *Oligopeptide repeats in the yeast protein Sup35p stabilize intermolecular prion interactions.* EMBO J., 2001. **20**(9): p. 2111-9.
47. Shkundina, I.S., et al., *The role of the N-terminal oligopeptide repeats of the yeast sup35 prion protein in propagation and transmission of prion variants.* Genetics, 2006. **172**(2): p. 827-35.
48. Cox, B.S., *PSI, a cytoplasmic suppressor of super-suppressor in yeast.* Heredity, 1965. **26**: p. 211-232.
49. Lancaster, A.K., et al., *The spontaneous appearance rate of the yeast prion [PSI+] and its implications for the evolution of the evolvability properties of the [PSI+] system.* Genetics, 2010. **184**(2): p. 393-400.
50. Wickner, R.B., *[URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae.* Science, 1994. **264**(5158): p. 566-9.
51. Derkatch, I.L., et al., *Genetic and Environmental Factors Affecting the de novo Appearance of the [PSI(+)] Prion in Saccharomyces cerevisiae.* Genetics, 1997. **147**(2): p. 507-519.
52. Ferreira, P.C., et al., *The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation.* Mol. Microbiol., 2001. **40**(6): p. 1357-69.
53. Jung, G. and D.C. Masison, *Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions.* Curr. Microbiol., 2001. **43**(1): p. 7-10.
54. Ness, F., et al., *Guanidine hydrochloride inhibits the generation of prion "seeds" but not prion protein aggregation in yeast.* Mol. Cell. Biol., 2002. **22**(15): p. 5593-605.
55. Paushkin, S.V., et al., *Propagation of the yeast prion-like [psi+] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor.* EMBO J., 1996. **15**(12): p. 3127-34.
56. Wegrzyn, R.D., et al., *Mechanism of prion loss after Hsp104 inactivation in yeast.* Mol. Cell. Biol., 2001. **21**(14): p. 4656-69.
57. LeVine, H., 3rd, *Quantification of beta-sheet amyloid fibril structures with thioflavin T.* Methods Enzymol, 1999. **309**: p. 274-84.
58. Lopez de la Paz, M. and L. Serrano, *Sequence determinants of amyloid fibril formation.* Proc Natl Acad Sci U S A, 2004. **101**(1): p. 87-92. Epub 2003 Dec 22.

59. Thompson, M.J., et al., *The 3D profile method for identifying fibril-forming segments of proteins.* Proc Natl Acad Sci U S A, 2006. **103**(11): p. 4074-8.
60. Chen, B., et al., *Genetic and epigenetic control of the efficiency and fidelity of cross-species prion transmission.* Mol Microbiol, 2010. **76**(6): p. 1483-99.
61. Santoso, A., et al., *Molecular basis of a yeast prion species barrier.* Cell, 2000. **100**(2): p. 277-88.
62. Tessier, P.M. and S. Lindquist, *Prion recognition elements govern nucleation, strain specificity and species barriers.* Nature, 2007. **447**(7144): p. 556-61. Epub 2007 May 9.
63. Pastor, M.T., A. Esteras-Chopo, and L. Serrano, *Hacking the code of amyloid formation: the amyloid stretch hypothesis.* Prion, 2007. **1**(1): p. 9-14. Epub 2007 Jan 5.
64. Teng, P.K. and D. Eisenberg, *Short protein segments can drive a non-fibrillizing protein into the amyloid state.* Protein Eng Des Sel, 2009. **22**(8): p. 531-6.
65. McGlinchey, R.P., D. Kryndushkin, and R.B. Wickner, *Suicidal [PSI+] is a lethal yeast prion.* Proc Natl Acad Sci U S A, 2011. **108**(13): p. 5337-41.
66. Ohhashi, Y., et al., *Differences in prion strain conformations result from non-native interactions in a nucleus.* Nature, 2010. **6**(3): p. 225-230.
67. Street, A.G. and S.L. Mayo, *Intrinsic beta-sheet propensities result from van der Waals interactions between side chains and the local backbone.* Proc Natl Acad Sci U S A, 1999. **96**(16): p. 9074-6.
68. Espinosa Angarica, V., S. Ventura, and J. Sancho, *Discovering putative prion sequences in complete proteomes using probabilistic representations of Q/N-rich domains.* BMC Genomics, 2013. **14**: p. 316.
69. Crow, E.T., Z. Du, and L. Li, *A small, glutamine-free domain propagates the [SWI(+)] prion in budding yeast.* Mol Cell Biol, 2011. **31**(16): p. 3436-44.

CHAPTER 3: WHY DO PRION DOMAINS EXIST?

Introduction

There are a number of theories as to why yeast carry so many prion-forming and prion-like domains. This chapter reports on two ongoing lines of investigation, designed to resolve this issue. First, a novel bioinformatics algorithm (GARRF) is used to screen a wide range of proteomes to find examples of Q/N-rich domains outside of *Saccharomyces cerevisiae*. Identifying other species that carry these unusual genomic quirks provides insight into their role in cellular biology. We find a wide range species carry prion-like domains at levels comparable to *Saccharomyces cerevisiae*.

Second, currently researchers rely primarily on yeast genetic methods to discover and monitor prions. These methods have a number of drawbacks, including a glacially slow readout time. This chapter reports on progress towards the development of a novel fluorescence based prion assay. This assay takes advantage of bi-molecular fluorescence complementation, a technique that uses complementary fragments of a fluorescent protein to indicate when two interacting domains are in proximity to one another. When completed, this assay will provide a means to monitor protein aggregations that is both faster and more sensitive than any existing assay.

Background

Despite more than 20 years of research and an increasingly sophisticated understanding of yeast prion physiology, the most fundamental question about yeast prions remains controversial. We still don't know *why* yeast carry prion-forming domains. There are two major schools of thought regarding the function of prion domains.

A number of labs, including those of Susan Lindquist, Randal Halfmann and Heather True, have published papers purporting to demonstrate that the prion state is part of a beneficial biological function [127, 132, 142]. They claim that prion-forming domains are conserved amongst closely related yeast, that the prion state is induced by stress, and that it mitigates stress conditions. Conversely, the Wickner laboratory has consistently published papers supporting the idea that prions are a disease state. They argue that the scarcity of wild prion infections, and the fact that several prion-forming domains have putative functions, indicates that prions are a disease state [126, 131].

Our work in the Ross lab generally focuses on the compositional and sequential requirements of prion formation, but some of the lab's recent findings speak to the issue of prion function. Recently, the Ross lab published a series of experiments exploring the mechanism by which Q/N rich non-prion domains might mutate into prion domains. They found that as few as two point mutations could convert a Q/N rich non-prion domain into a prion domain [141]. This finding meshes well with recent work from the Tartaglia lab.

Tartaglia's group found that proteins are prone to aggregation outside the fairly narrow range of their normal environment and expression level. They hypothesize that this is a natural consequence of evolution. Uncontrolled protein aggregation is deleterious, and therefore strongly selected against. However, once a domain is soluble under normal physiological conditions, there is no further driving force improving protein solubility. So proteins will evolve just to the point of solubility under normal physiological conditions. Because most point mutations increase the aggregation propensity of a given protein, over time a protein will tend to evolve to a state where it is just barely stable. They use this "life on the edge" hypothesis to explain why yeast might carry these problematic domains [140].

Progress Towards a Novel Yeast Prion Assay

A major obstacle to resolving these issues is the nature of the experimental tools available to monitor prion formation. To date, the majority of yeast prion research is carried out using yeast genetics based assays. These assays provide a readout after a minimum of three days of cell growth, and some assays require up to seven days of growth. This precludes the study of any phenomena that occurs on a shorter time scale

In order to study prions in real-time, a faster assay needs to be developed. A number of groups have looked at yeast prions using GFP-labeled prion proteins [2, 212-214]. This method has been useful for identifying the presence of aggregated prion proteins in yeast cells, monitoring prion particle size using FRAP and documenting mother-daughter prion transmission using live-cell imaging [215].

However, conventional fluorescent labeling of prion proteins has technical limitations, most notably, detection of prion aggregation requires that a significant portion of the prion protein is aggregated, enough that the aggregate signal can be resolved from the background fluorescence of soluble protein. Most prions only consume a fraction of the available monomeric protein, leaving significant background fluorescence.

This chapter focuses on progress towards developing a novel fluorescence-based prion assay. This researcher found that a bi-molecular fluorescence complementation assay (BiFC) filled many of the gaps left by other experimental methods. A BiFC assay is based on structural complementation between two fragments of a fluorescent protein, in this case the venus fluorescent protein. The venus fluorescent protein has been subdivided into two protein fragments, neither of which fluoresces on its own. These protein fragments are each fused to one

of two proteins of interest. If the proteins of interest interact, the two halves of the fluorescent protein are brought into close proximity and can reconstitute a functional fluorophore.

The N-terminal domain of Ure2 prion domain is sufficient to nucleate and maintain a prion infection [216]. Indeed, yeast prion domains are modular in that other sequences can be attached to them and they still form amyloid [57, 217]. This assay uses this property to design a BiFC-based amyloid detection assay. Both halves of a BiFC fluorophore will be fused to the Ure2 prion-forming domain (residues 1-89). As amyloid fibers nucleate and grow *in vivo*, these reporter proteins will be incorporated into growing fibers. If two complementary BiFC fusion proteins are incorporated next to one another, the BiFC moieties will reconstitute and fluoresce.

BiFC has the advantage of generally producing a higher signal-to-noise ratio than conventional fluorescent labelling, as the BiFC fluorophore only forms during protein-protein interactions. A BiFC assay also has the advantage that it can be used in fluorescence-activated cell sorting (FACS) to screen through a culture of cells and identify prion specimens.

Technical Limitations of a BiFC Assay

In theory, a BiFC assay could be used to detect prion nucleation via live-cell imaging. Recent advances in single molecule-spectroscopy allow for the detection of individual fluorophores using epifluorescent microscopes [218]. However, technical limitations specific to yeast make this unlikely. Yeast are fairly small relative to other eukaryotes, approximately 4-6 microns in diameter, and the outer layer of their cell wall is a thick coating comprised of β-glucans, chitin, mannose and proteins. Yeast also autofluoresce, reducing the signal-to-noise ratio to a point that singe-molecule spectroscopy may not be possible. Yeast make for a powerful experimental system, but they are not usually chosen for their amenability to microscopy.

At this time, the exact sensitivity of the assay is not known, but, prion physiology gives us some insight into the factors that will determine assay sensitivity. The sensitivity of the assay will depend largely on the level of expression of the BiFC fusion proteins. More copies of the BiFC fusion proteins increases the likelihood that two fusion proteins will be incorporated next to each other in a growing amyloid fiber, increasing the signal strength. Unfortunately, over-expressing the prion-forming domain dramatically increases the rate of prion formation and de-stabilizes existing prions [219]. A key aspect to optimizing this assay will be finding a level of expression that allows for the detection of amyloid, without interfering with prion physiology.

At the time that this dissertation is written, more work will still be required to develop this assay. However, early experiments, presented in this chapter, demonstrate that BiFC fragments attached to a Ure2 prion domain incorporate into amyloid fibers *in vivo* to generate a fluorescence signal. This finding provides the basis for an assay to measure prion aggregation in yeast cells. Unfortunately, the plasmid vector used to express these proteins does not propagate consistently, rendering the system unsuitable for most experiments. This chapter describes progress towards a working assay, diagnoses issues with the existing assay, and provides direction for future work.

Bioinformatics studies of Q/N rich domains across many species

Another approach to decoding the function of yeast prion domains is to understand their context relative to other genomes. The yeast genome contains at least a dozen prions, and hundreds of domains that are compositionally similar to Q/N-rich prion domains. The large number of yeast prions may be related to the fact that the yeast genome is unusually dense with Q/N-rich domains. Researchers Michelitsch and Weissman surveyed the Q/N-rich domains of a small set of species using a simpler algorithm that flagged a proteins as having at least one Q/N

rich domain [95]. They found that eukaryotes have a higher percentage of proteins with at least one Q/N rich domain than prokaryotes. However, due to the limited number of species with mapped genomes at the time of publication, their survey didn't contain many higher organisms.

There are several possible mechanisms by which natural selection can generate Q/N-rich domains. If Q/N-rich domains are an artifact of natural genetic drift, or the remnants of entropically decomposing polyglutamine expansions, one would expect them to be roughly evenly distributed throughout all species. If they represent an adaptation to a specific environment, they will be tolerated by specific species, or clades. In order to better understand how common Q/N-rich domains are, this researcher developed an algorithm GARRF (Glutamine/Asparagine Rich Region Finder) that scans through a proteome and returns domains with Q+N content above a user-defined threshold. The GARRF algorithm was used to scan the proteomes of 30 species, taken from a wide range of phyla.

GARRF operates by scanning for any consecutive domain, larger than 40 residues, with more than 30% glutamine and asparagine residues. The Q/N-rich content was calculated by dividing the total length of all Q/N-rich domains by the total length of the proteome.

We found that Q/N-content is higher in celled-yeast, soma single-celled eukaryotes, and the fruit fly. There as a wide range of Q/N content. Some species tolerate levels as high as 2% (*Paramecium tetraurelia*) and others as low as 0% (the fungus, *Encephalitozoon cuniculi*).

Materials and Methods

Media and Strains

Standard yeast media and methods were used, as described previously [220]. Yeast were incubated at 30°C. Liquid cultures were incubated at 30°C, with shaking at 100-200 rpm All BiFC experiments were carried out in YER135 (*MAT***a** *Δura2 Δleu2 Δtrp1*) strain.

Constructs and Cloning

A total of five constructs were generated each linking a VN or VC moiety to either the C or N terminus of the Ure2 prion domain, separated by a GCGGCG linker-domain (see Figure 3.1). The VC (residues 1-173) and VN (residues 155 – 238) moieties were amplified from plasmids provided by Dr. Chaoping Chen using oligomers EDR951, EDR952, EDR953, EDR954, EDR957, EDR958, ERD959 and EDR960 (See Table 3.1 for the VN and VC sequence and 3.2 for a list of all oligonucleotides). The sequence encoding the Ure2 prion forming domain (residues 1-89) was amplified from plasmid pER62 using oligonucleotides EDR1001, EDR950, EDR955 and EDR956. The venus moieties were fused to the PFD domains by co-amplifying them with oligonucleotides EDR68, EDR954, EDR959, EDR956, EDR960 and EDR956. The end-products of these reactions were digested with BamHI and XhoI and ligated into pER41 (*TRP1*) and pER56 (*LEU2*), 2μ plasmids derived from pRS424 containing a *GAL1* promoter, an *ADH1* terminator and an Amp1 marker.

All constructs were appended with HA2 tags by amplifying the constructs with a GSGGSG linker at their 3' end using oligonucleotides EDR1135, 1136, EDR1101, EDR1102 EDR1103, EDR1120 (see Table 3.2 for a description of the oligonucleotides used). The products of these reactions were used as templates for a PCR reaction that appended the constructs with an HA2 epitope tag using oligonucleotides EDR1101, EDR1102, EDR1103 and EDR1056. The end-products of these reactions were digested with BamHI and XhoI and ligated back into pER41 and pER56.

Microscopy and Fluorescence Activated Cell Sorting and Analysis

All microscopy was performed on a Nikon Diaphot 300 epifluorescent microscope at 20, 40 and 100x magnification. Cells were fixed and mounted using Invitrogen Prolong Gold

antifade reagent. All cell sorting and analysis were performed on a MoFlo Flow Cytometer and High Speed Cell Sorter (Dako Colorado, Inc.).

Plasmid Loss Measurements

Cultures were grown for 24 hrs under selective conditions (10 ml SC-Trp-Leu media). Then cultures were spun down, decanted and re-suspended in both non-selective glucose and galactose media at an optical density of OD600 0.05. Cultures were grown for 48-72 hrs. and samples were taken from the media at five time points. Samples were plated to both selective and non selective media to measure the rate of plasmid loss

Bioinformatics

The bioinformatics script (GARRF) was written in Python (see Addendum 2). All computations were performed on an Apple Macbook Air. A Python-based FASTA file parser was provided by Dr. Asa Ben-Hur.

Table 3.1 The sequence of VN and VC provided by Dr. Chaoping Chen.

VN	atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccg gcgagggcgagggcgatgccacctacggcaagctgaccctgaagctgatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtga ccaccctgggctacggcctgcagtgcttcgcccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtc caggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgag ctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcaccgccgaca agcagaagaacggcatcaaggccaacttcaagatccgccacaacatcgagtag
VC	gacaagcagaagaacggcatcaagctgaacttcaagatccgccacaacatcgaggacggcggcgtgcagctcgccgaccactaccagcagaac acccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagctaccagtccgccctgagcaaagaccccaacgagaagcgcgat cacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaagtaa

Table 3.2 Primers used to generate VN and VC fusion constructs.

Description	Oligomer	Sequence
Sense primer binding to WT Ure2 at 1 and amending with a His tag and a BamH1 site.	EDR1001	GCAGCAggatccATGcaccaccaccaccaccacATGATGAATAACAACGGCAACCAAGTGTCG
3' anti-sense primer binding at the end of the Ure2PD amending with a serine-glycine linker	EDR950	accggaaccggaaccggaCTGTTGTTGTTGTCGATGTTGTTCTAAGG
5' sense primer binding VN, amended with a poly-serine-glycine linker	EDR951	ggttccggtggttccggtATGGTGAGCAAGGGCGAGGAGC
5' sense primer binding VC, amended with a poly-serine-glycine linker	EDR952	GACAAGCAGAAGAACGGCggttccggtggttccggt
3' anti-sense primer binding at the end of VN adding a stop codon and an Xho1 restriction site	EDR953	gacgacCTCGAGctaCTACTCGATGTTGTGGCGG
3' anti-sense primer binding at the end of VC adding a stop codon and an Xho1 restriction site	EDR954	gacgacCTCGAGctaTTACTTGTACAGCTCGTCCATGC
5' sense primer binding to the Ure2PD amending with a poly-serine-glycine linker	EDR955	ggttccggtggttccggtATGATGAATAACAACGGCAACCAAGTGTCG
3' anti-sense primer amending Ure2PD with a stop codon and an Xho1 restriction site	EDR956	atgatgCTCGAGctaCTGTTGTTGTTGTCGATGTTGTTCTAAGG
3' anti-sense primer binding the VN and amending with a poly-serine-glycine domain	EDR957	accggaaccggaaccggaCTCGATGTTGTGGCGGATCTTGAAG
3' anti-sense primer binding the VC and amending with a poly-serine-glycine domain	EDR958	accggaaccaccggaaccTTACTTGTACAGCTCGTCCATGCC
5' sense primer binding to VN amending with a Bam1 site a start codon and a His tag	EDR959	GCAGCAggatccATGcaccaccaccaccaccacATGGTGAGCAAGGGCGAGG
5' sense primer binding to VC amending with a Bam1 site a start codon and a His tag	EDR960	GCAGCAggatccATGcaccaccaccaccaccacGACAAGCAGAAGAACGGC
Anti-sense primer binding to the 3' end of VN and amending with a GSGGSG linker	EDR1135	ACCgctTCCTCCAGAACCCTCGATGTTGTGGCGGATCTTGAAG
Anti-sense primer binding to the 3' end of VC and amending with a GSGGSG linker	EDR1136	ACCgctTCCTCCAGAACCCTTGTACAGCTCGTCCATGCC
Antisense contains GSGGSG anneals to 1120, 957, 958 amends with an HA tag , stop codon and an Xho1 site	EDR1056	gagatCTGCAGctcgagtttaTGCGTAATCTGGAACGTCGTAGggataGCCggcatagtctgggacgtcatatgggtaACCgctTCCTCCAGAACC
Sense primer binding to the 5' end of VN adding a start codon a BamH1 site and a linker domain	EDR1101	gcattagccatggtcacacgGGATCCATGGTGAGCAAGGGCGAGG
Sense primer binding to the 5' end of VC adding a start codon a BamH1 site and a linker domain	EDR1102	gcattagccatggtcacacgggatccATGGACAAGCAGAAGAACGGC
Sense primer binding to the 5' end of Ure2 adding a start codon a BamH1 site and a linker domain	EDR1103	gcattagccatggtcacacgGGATCCATGATGAATAACAACGGCAACCAAGTGTCG
Anti-sense primer binding to the 3' end of the Ure2 PD (residue 89) and amending with a GSGGSG linker	EDR1120	gtaACCgctTCCTCCAGAACCctgttgttgttgtcgatgttgttc

Results

BiFC Construct Design

We do not have a precise three-dimensional structure of the [URE3] amyloid. However, low-resolution structural techniques provide some insights as to the optimal BiFC arrangement. TEM imaging of amyloid fibers grown *in vitro* reveal that although amyloid fibers do come into contact with one another, they don't bundle together in any systematic way [43, 221]. Also, SSNMR studies show that yeast prions form parallel in-register ß-sheets. Based on this data, it was determined that the best chance for an interaction in the amyloid state is to have both the VC and VN moiety placed on the same terminal end of the Ure2 prion-forming domain.

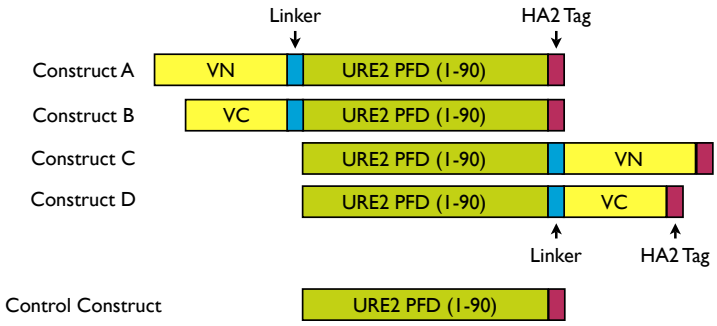


Figure 3.1 BiFC Fusion Protein Diagram
A diagram of the five BiFC constructs. Venus BiFC moieties (yellow) are joined to URE2 prion forming domains (green) by way of a GSGGSG linker (blue). All constructs are labeled with an HA2 tag at their C-Terminus (red).

In order to find the optimum BiFC orientation, four constructs were generated, two with VN and VC moieties attached to the N-terminus of the Ure2 prion domain, and two with VN and VC moieties attached the C-terminus of the Ure2 prion domain (see Figure 3.1). The VN and VC moieties were separated from the Ure2 prion domain by a GSGGSG sequence and each construct is labeled with a C-terminal HA label. The HA label is also attached with a GSGGSG linker. A fifth construct, containing only the Ure2 prion domain with a C-terminal HA label, was also constructed.

De novo prion formation is a rare event, infecting approximately one in a million cells. Transient over-expression of the prion domain of the Ure2 gene has been shown to dramatically increase prion formation [50]. In order to ensure a measureable rate of prion formation, constructs were sub-cloned into high-copy plasmids (2µ shuttle plasmids) under the control of the *GAL1* promoter and the *ADH1* terminator. Specifically, the VN fusion constructs were sub-cloned into pER41, a shuttle plasmid carrying the *TRP1* auxotrophic marker, and the VC fusion constructs were sub-cloned into pER56, a shuttle plasmid carrying the *LEU2* auxotrophic marker. A copy of the HA-labelled Ure2 prion domain was sub-cloned into a copy of both pER41 and pER56, as a non-BiFC control.

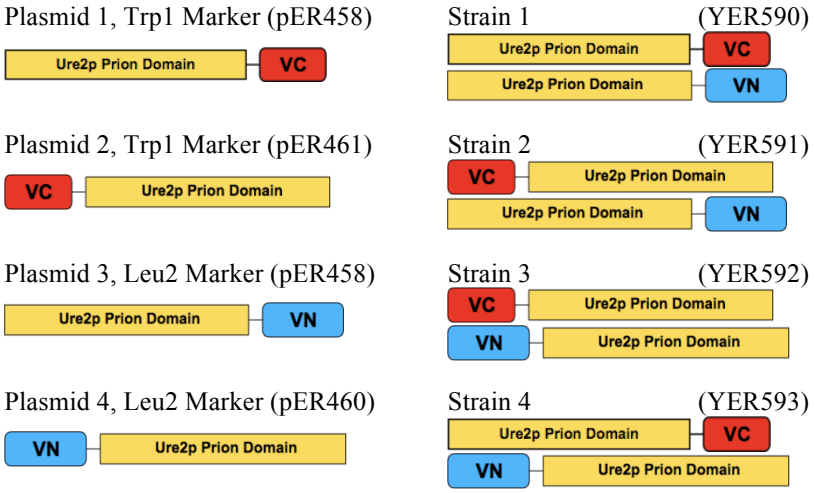


Figure 3.2 Plasmid and Yeast Strain Diagram
A diagram of the plasmid and strain scheme. All constructs were sub-cloned into 2µ plasmids under the control of the GAL1 promoter and an ADH1 terminator. VC fusion plasmids carry a TRP1 marker and the VN fusion plasmids carry a LEU2 maker. Pairs of plasmids were transformed into a Δura2 Δleu2 Δtrp1 strain as shown. Strain one and three place the VN and VC moieties on the same end of the URE2 prion domain. Strain two and four, intended to act as negative controls, have the VN and VC on opposite ends of the URE2 prion domain.

Complementary pairs of VN and VC plasmids were transformed into the yeast strain YER135 in the manner shown in Figure 3.2.

Presumably, the VC and VN moieties can interact with one another independently of amyloid formation. Thus, it would be desirable to have a control to measure non-amyloid venus

fluorescence. Unfortunately, the co-expression of the VN and VC moieties by themselves provide a poor control, as the expression level and subcellular localization of the VC and VN protein fragments are often different from their fusion proteins. In many cases the fluorescent signal resulting from co-expression of the VC and VN fragments is actually greater than the fluorescence resulting from the co-expression of the fusion proteins [222]. A better approach is to attach the VN and VC reporters to two non-interacting proteins.

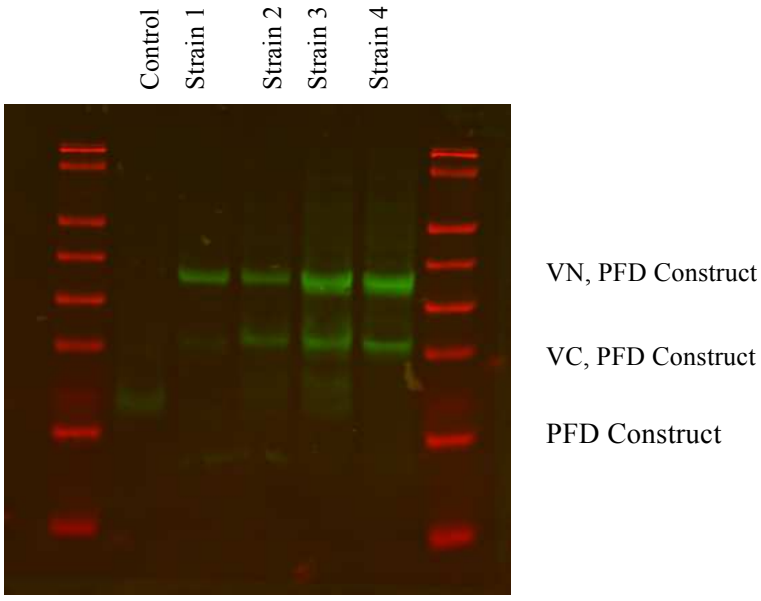


Figure 3.3 BiFC Fusion Protein Expression *In Vivo*
A western blot of the four strains and the control strain. All constructs expressed. The VC construct in strain 1 does express at a slightly lower intensity than the other constructs.

To provide controls, two strains were generated that carried the VN and VC fusion proteins attached to opposing ends of the Ure2 prion domain (see Figure 3.2, strains 2 and 4). It was initially assumed that these configurations would result in little or no fluorescence signal as the only VC and VN interaction would occur from non-amyloid interactions and from intra-fiber interaction.

A western blot, using anti-HA2 antibodies, revealed that all constructs were expressing the appropriate length construct when cultured in the presence of galactose (see Figure 3.3), There were slight differences in the expression levels of the constructs. Notably, the VC fusion construct from strain 1 showed weaker expression than the other constructs.

It is possible that the VN/VC interaction could drive prion nucleation and thus increase the rate of prion formation. It's also possible that the VN/VC interaction could disrupt the chaperone-mediated severing of prions, disrupting their ability to propagate. In order to test these possibilities, the rate of *de novo* prion formation was measured for all five strains. Strains were cultured in synthetic complete galactose/raffinose –Leu -Trp media for approximately 72 hours. Serial dilutions of the cultures were plated to synthetic dropout +USA plates, conditions that are selective for [URE3] (See Figure 3.4). All strains formed prion within roughly an order of magnitude of the control strain, indicating that the presence of the venus moieties do not significantly affect the rate of prion formation. Colonies from each SD+USA plate were re-streaked to SD+USA plates to identify stable strains. All four strains were capable of generating prions that propagate past three consecutive rounds of re-streaking, indicating that these strains are capable of hosting stable [URE3] infections (data not shown).

BiFC Fluorescence reveals cellular aggregation of Ure2p

BiFC Strains were cultured in synthetic dropout –leu –trp glucose media (non-expression conditions) and synthetic minimal + ura galactose/raffinose media (fusion protein expression conditions) for approximately 48 hrs. All five strains were also cultured in synthetic minimal + USA 2% galactose /1% raffinose media (expression conditions selective for the [URE3] phenotype). These cultures grew slowly and required 120 hrs to reach a cell density adequate for FACS analysis. Cultures were analyzed via FACS (see Figure 3.5). Strains cultured in glucose media show a single population of cells with a roughly normal distribution of fluorescent intensity. The distribution of fluorescent intensity of the non-BiFC control strain is slightly lower than that of the BiFC strains, hinting that there may be some leaky expression of the venus fusion proteins. When cultured in galactose media, all of the strains develop a second sub-

population of cells with higher fluorescence intensity. Strain 1 has a distinct bi-phasic distribution of fluorescent intensity, while the others are a little more complicated. This is interesting as strains 2 and 4 were intended to serve as negative controls, with their BiFC domains attached to opposite ends of the prion forming domain. When cultured in media selective for [URE3], the high-intensity sub-population increases in strains 1 and 2, and is marginally reduced in strains 3 and 4. Of the four strains, strain 1 consistently showed the clearest bi-phasic fluorescence distribution during FACS analysis. It was used for all future work.

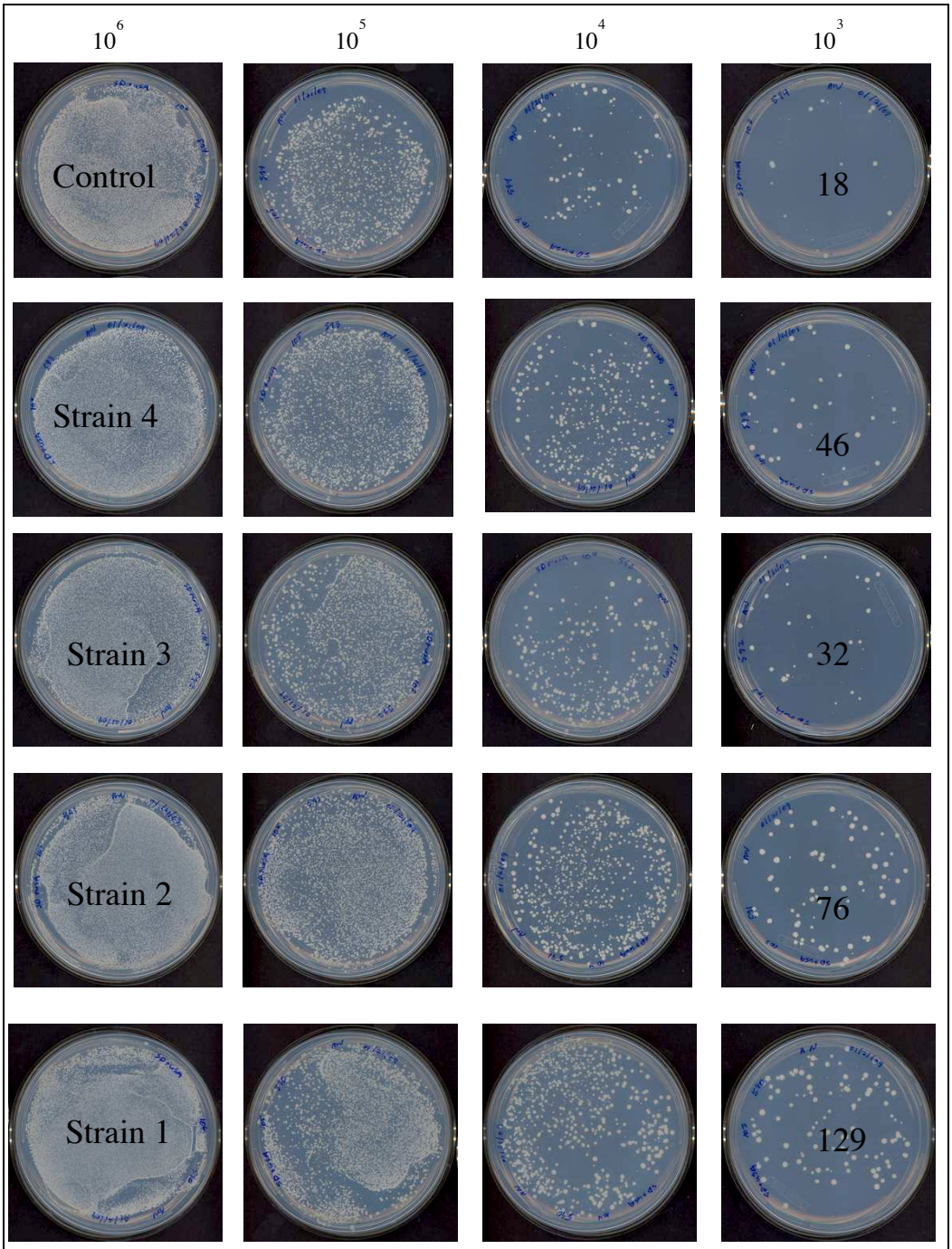


Figure 3.4 Rate of *De Novo* Prion Formation in BiFC Strains
Strains were tested for the rate of *de novo* prion formation. All strains were cultured for 72 hours in synthetic complete galactose/raffinose –Leu -Trp media. Cultures were diluted to normalize cell density and serial dilutions were plated to synthetic dropout +USA media. All strains formed within an order of magnitude of one another.

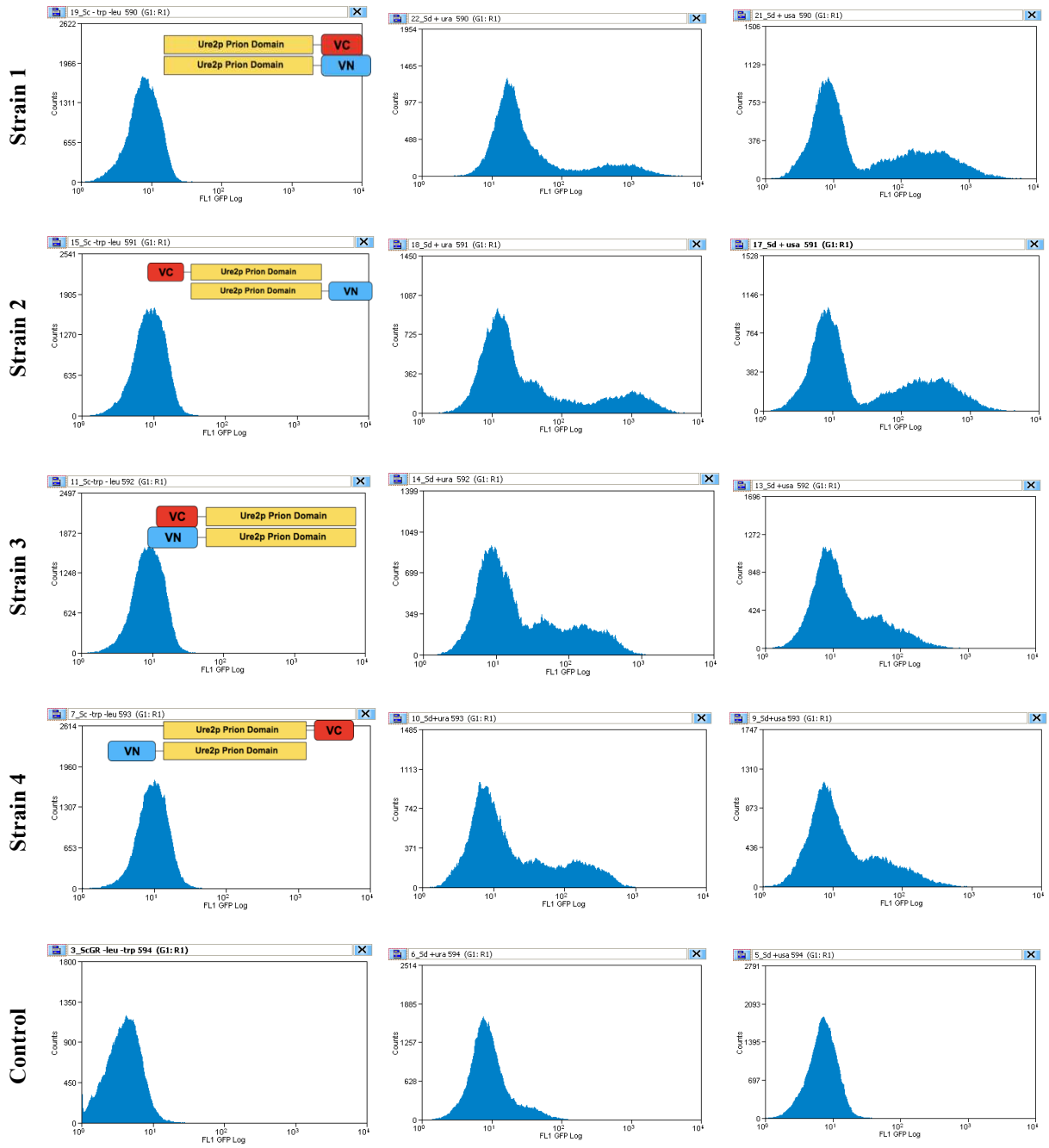


Figure 3.5 FACS Analysis of BiFC Strains
Strains cultured in synthetic dropout –Leu -Trp and synthetic minimal +ura galactose/raffinose media for 48 hours. Synthetic Minimal + USA galactose/raffinose media required approximately 120 hrs to reach adequate cell density for FACS analysis.

Microscopy

Unsorted cells from Strain 1 were observed under an epifluorescence microscope, after approximately 72 hrs in synthetic minimal + ura galactose/raffinose media (Figure 3.6). Cells showed two phenotypes. Some cells had amorphous concentrations of fluorescence of varying sizes. These fluorescent agglomerations appeared in seemingly random locations throughout the cell. Most cells carried a single agglomeration, but a few (approximately 10%) had either two or three per cell. Other cells showed low intensity background fluorescence evenly distributed throughout the cell.

Cells from strain 1 were sorted as shown in Figure 3.7A. Cells taken from the first low-intensity fluorescent population were observed under an epi-fluorescence microscope. None of the cells observed showed fluorescent protein aggregation (see Figure 3.7B). Cells taken from the high intensity peak carried amorphous agglomerations in approximately 95% of the cells observed (See Figure 3.8).

Sorted cells from strain 3 were plated to

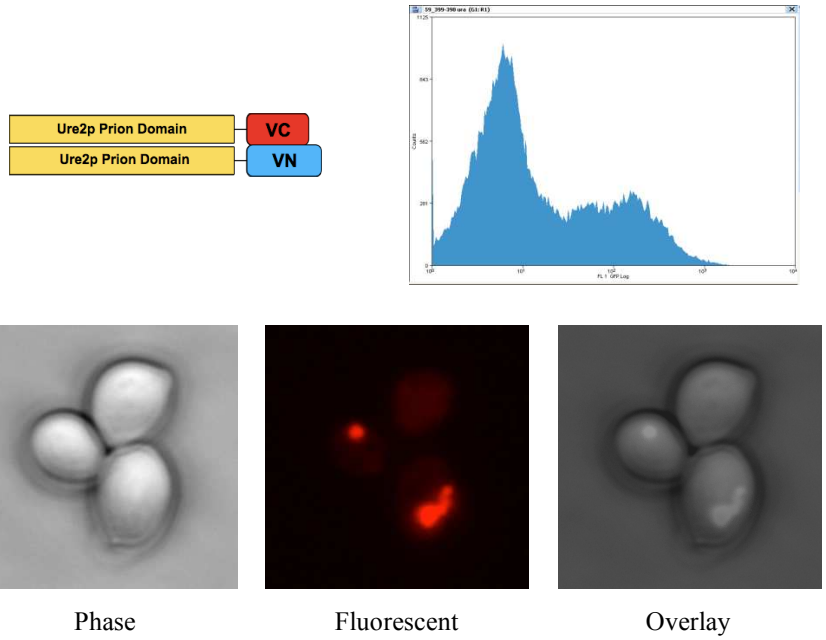


Figure 3.6 Epifluorescence Microscopy of a BiFC Strain
Cells from strain 1 were cultured in synthetic minimal + ura galactose/raffinose media for 48 hours before FACS analysis and microscopy. A diagram of the BiFC fusion proteins in strain 1 (top left). A FACS histogram of strain 1 (top right). An image of yeast cells taken at 100x in phase (bottom left), fluorescent (bottom middle) and overlay (bottom right).

YPAD plates (non-selective conditions) and incubated for three days. The resulting colonies were replica plated to synthetic minimal USA plates (conditions selective for the [URE3] phenotype). None of the colonies taken from the non-aggregate fraction were [URE3] (out of a total of 402 colonies). 17% of the colonies from the positive fraction were [URE3] (60 out of 353 colonies) (data not shown).

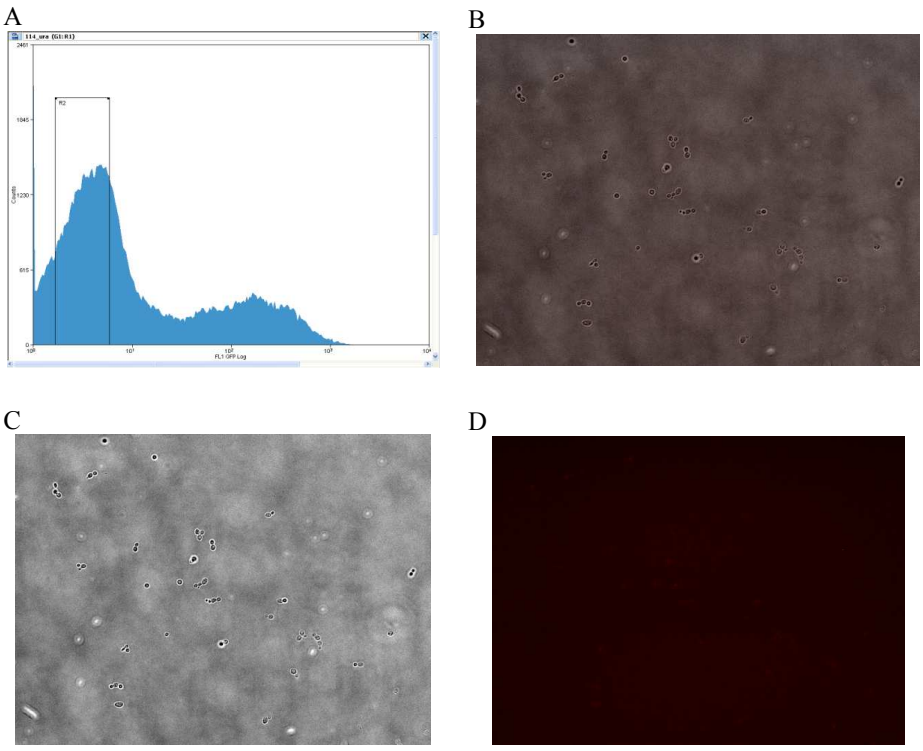


Figure 3.7 Epifluorescence Microscopy of Sorted BiFC Strains
Cells from strain 1 were cultured in synthetic minimal + ura galactose/raffinose media for 48 hours before fluorescent activated cell sorting. **A.** A FACS histogram of strain 1 showing the range of sorted cells. Cells inside the bracket were segregated and observed under an epifluorescence microscope. **B.** An overlay of the phase and fluorescence images at 10x magnification. **C.** Phase image of sorted cells. **D.** A fluorescence image of the cells. None of the cells showed any fluorescent agglomeration, but they all showed diffuse background fluorescence.

Plasmid Instability

During the execution of a number of experiments, strain 1 slowly lost fluorescence signal during long periods of culture (see Figure 3.9 for an example). This happened in all cultures, but it occurred more quickly in non-selective conditions. This could be because the prion variants are unable to consistently propagate from mother to daughter cells.

In order to identify the source of the strain instability, a culture of strain 3 was grown for approximately 72 hrs in synthetic minimal + ura galactose/raffinose media. The culture was sorted into four vials, segregating the prion-negative peak and dividing the prion-positive peak into three fractions (see Figure 3.10). The cells from these fractions were plated out to YPAD (non-selective media) and grown for three days. These plates were replica-plated to synthetic minimal + USA media (screening for prions), and synthetic complete -Trp -Leu (testing for loss of the plasmids). As can be seen in Figure 3.10, the "negative" cells were actually yeast that had lost one or both of the plasmid vectors, and thus weren't expressing the BiFC constructs at all.

There are several possible reasons why these cultures lose their plasmids. It could be that the plasmid is faulty, or that the presence of amyloid fibers interferes with segregation of 2μ plasmids. In order to test if the rapid plasmid loss was caused by BiFC

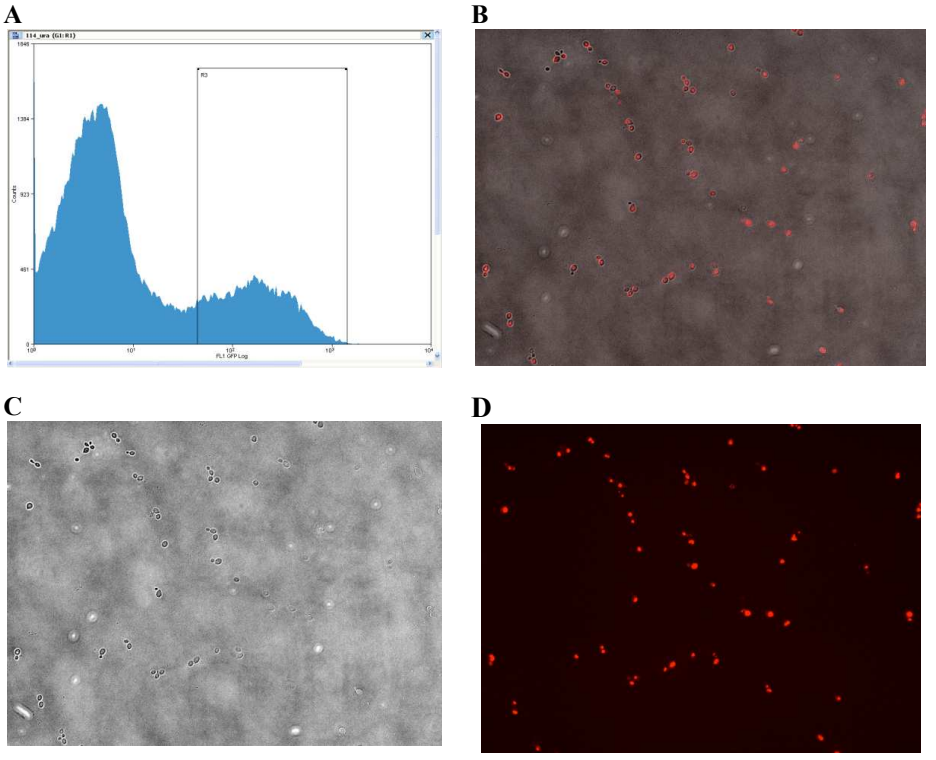


Figure 3.8 Epifluorescent Microscopy of Sorted BiFC Strains
Cells from strain 1 were cultured in synthetic minimal + ura galactose/raffinose media for 48 hours before fluorescent activated cell sorting. **A.** A FACS histogram of strain 1 showing the range of sorted cells. Cells inside the bracket were segregated and observed under an epifluorescent microscope. **B.** An over lay of the phase and fluorescent images at 10x magnification. **C.** Phase image of sorted cells. **D.** An image of the fluorescent cells. Approximately 95% of all cells showed one to three fluorescent agglomerations randomly distributed throughout the cell.

construct expression, strains 1 and the control strain (expressing the Ure2 PFD) were cultured separately in galactose and glucose media for 54 hours (see Figure 3.11). Samples were pulled at five time points and plated to conditions that select for the presence of plasmids.

The cultures grown in galactose clearly lose the plasmid faster than those grown in glucose. But surprisingly, even the glucose-grown cultures seem to reach a steady state of plasmid occupancy around 40%. It is also interesting to note that the cultures all start around 60%, indicating that there is significant plasmid loss even under selective conditions. This experiment was repeated on the control strain, carrying only the Ure2 prion domain, to see if the

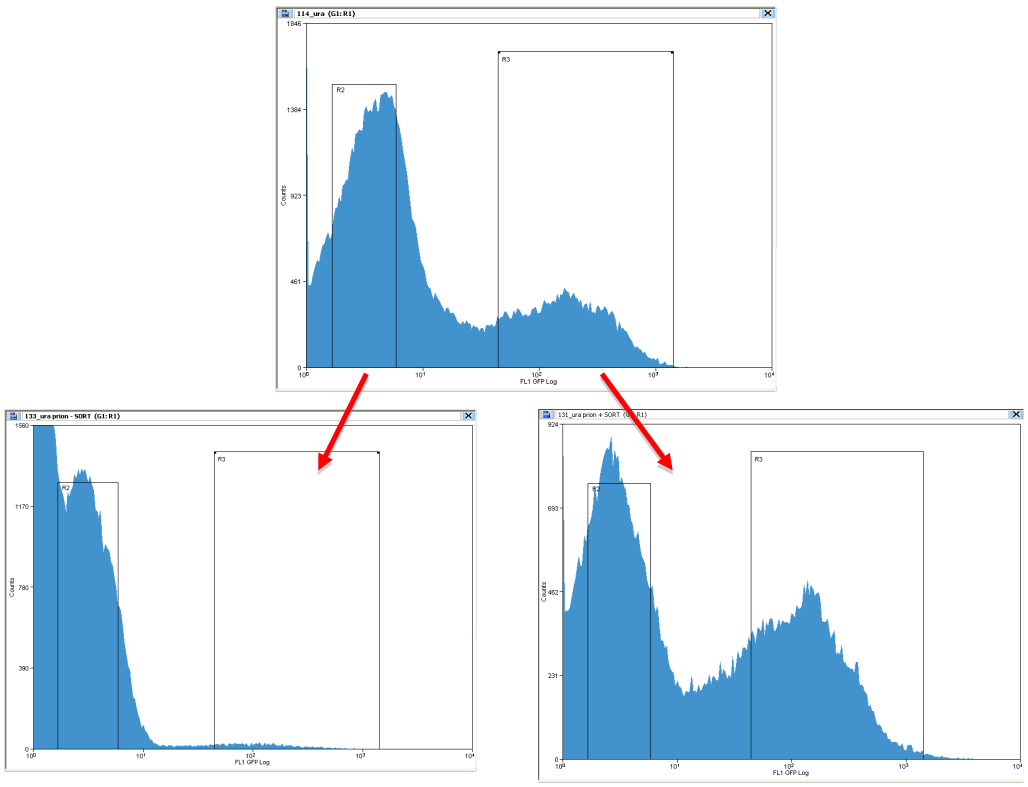


Figure 3.9 Prion Stability Monitored by Fluorescent Activated Cell Sorting
Strain 1 was cultured in synthetic minimal + ura galactose/raffinose media for three days. The culture was sorted using the gating shown in on top. Cultures were inoculated with cells from the two fractions, incubated for another three days, and analyzed via FACS again. The prion fraction (on the right) lost a significant amount of its fluorescence over the course of three days of growth. Cells from the non-prion fraction (on the left) didn't convert to the prion state at an appreciable rate.

presence of BiFC moiety is somehow disrupting the plasmid's ability to propagate. The control strain behaved similarly to strain 1. When cultured under selective conditions in glucose for 72 hours, about 70% of the culture carries the plasmid. When cells were transferred to non-selective media the plasmid occupancy drops to about 40%, and in galactose media vector occupancy dropped to 10% (data not shown).

The 2μ plasmid is a naturally occurring, benign parasite that uses the cell's native spindle separation mechanism to ensure successful plasmid propagation from mother to daughter cells. The wild-type 2μ-plasmid encodes four genes, *REP1, REP2, STB* (sometimes called *REP3*), and *FLP1*.

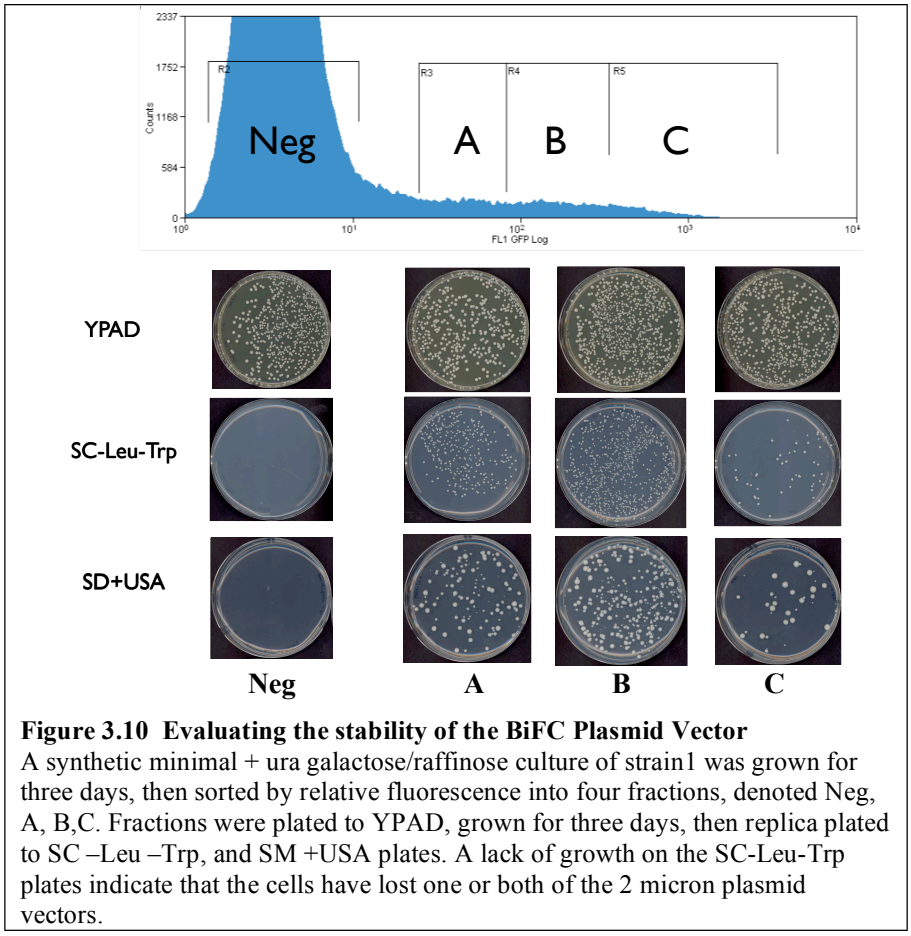


Figure 3.10 Evaluating the stability of the BiFC Plasmid Vector
A synthetic minimal + ura galactose/raffinose culture of strain1 was grown for three days, then sorted by relative fluorescence into four fractions, denoted Neg, A, B,C. Fractions were plated to YPAD, grown for three days, then replica plated to SC –Leu –Trp, and SM +USA plates. A lack of growth on the SC-Leu-Trp plates indicate that the cells have lost one or both of the 2 micron plasmid vectors.

The *REP1* and *REP2* genes are cis-acting proteins that bind to the STB and each other, bundling the 20-60 copies of the 2μ-plasmid into 1 - 3 loci in the nucleus. During mitosis, this bundle acts like a crude chromosome, binding to the spindle axis. As the cell divides, the bundle is pulled into both the mother and daughter cells, ensuring equal or near equal segregation of the plasmid. The 2μ-plasmid is a remarkably successful parasite; indeed, it is a challenge to isolate a wild strain of *S. cerevisiea* that doesn't carry the plasmid.

The two parent plasmids used to generate these vectors are derived from the pRS42x family of 2μ-shuttle plasmids. These plasmids utilize the STB and FLP1 genes, combined with a bacterial promoter and an auxotrophic marker.Note that these plasmids lack the genes encoding the Rep1p and Rep2p proteins. They can only successfully propagate in cells that are cir^{+}, cells that carry a wild-type copy of the 2μ-plasmid. 2μ-plasmids are reported to be quite stable with a rate of plasmid loss of <2% per doubling [223].

Both plasmids were created by cloning a cassette carrying a *GAL1* promoter, an MCD domain, and an *ADH1* terminator into the MCD domain of the pRS424 and pRS425 plasmids. These parent plasmids were obtained from two different labs. It is worth noting that these plasmids have been used in research that lead to several publications.

It is possible that the yeast strain was either cir^{0}, or carrying a defective copy of the wild-type 2μ-plasmid. This would render it incapable of propagating 2μ-plasmids. It is also possible that the YER135 strain picked up a mutation that disrupts chromosomal segregation. This mutation could inhibit proper segregation of the pRS-based plasmids. In order to test this, another 2μ-plasmid (YEP181) was transformed into YER135 and the stability of the plasmid was monitored as described above. The plasmid maintained greater that 95% occupancy for more that 48 hrs (data not shown), indicating that the yeast strain is capable of propagating a 2μ-plasmid.

Taken together these results indicate that this plasmid will not work as a vector for the BiFC assay. The parent plasmid does not consistently propagate from mother to daughter during replication. Propagation fidelity is further reduced when the BiFC fusion proteins are expressed.

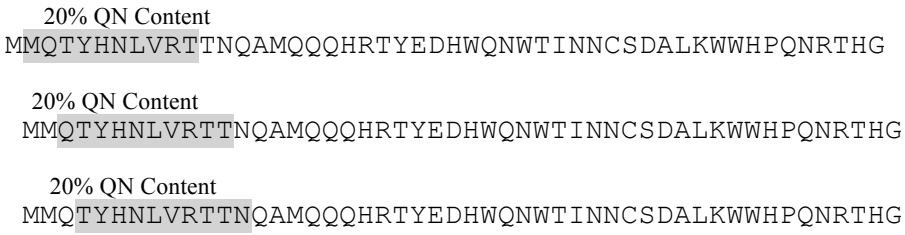
Compositional Biases in Glutamine/Asparagine Rich Domains

In order to identify regions of the proteome with unusual compositional biases, we developed an algorithm that scans through a genome and identifies Q/N-rich domains. Glutamine Asparagine Rich Region Finder (GARRF) scans through a protein one residue at a time recording the Q/N content within a 10-residue window. When it finds 10 residues with a user-defined minimum Q/N content, it jumps to the adjacent 10-residue window and measures its Q/N content. It keeps scanning through a protein in 10-residue blocks until it reaches a block in which Q/N content falls below the user-defined minimum. The resulting contiguous domain is recorded in a data file for future analysis (see Figure 3.12 for a description of the algorithm). Truncation studies indicate that the minimum length required for prion formation is about 40 residues, therefore we restricted our search to domains that are at least 40 residues long [60] . In order to avoid tri-nucleotide repeat domains, GARRF was set to discard any domains where the glutamine:asparagine ratio is greater that 1:5 or less than 5:1.

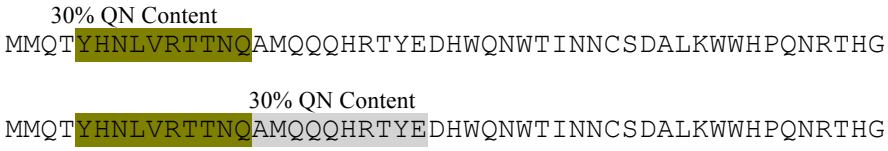
GARRF was used to scan through the complete proteomes of 30 species ranging from bacteria through *Homo sapiens*. The total length of all of the Q/N rich domains is reported as a percent of the total length of the proteome in Figure 3.13. We find that *Saccharomyces cerevisiae* is not particularly enriched with Q/N content when compared with other species. Indeed, paramecia carry an order of magnitude more Q/N -rich sequence. Furthermore Q/N-content seems to be centered around single celled-yeast, single-celled eukaryotes, and the fruit fly.

1. **GARRF queries a 10-residue window, measuring Q and N content.**

2. **If the QN content is below a given threshold (30%), GARRF shifts the window by one residue (n+1) and tests again.**



3. **If the threshold is breached, the ten residue window is flagged as QN rich and the next ten residue window (n+10) is analyzed.**



4. **If this window is also greater than the threshold, it is added to the QN rich domain and GARRF jumps downstream by another ten residues (n+10).**

5. **This continues until the QN content falls below the given threshold. GARRF adds the QN rich domain to a list and returns to scanning through the sequence, till another QN rich domain is acquired.**

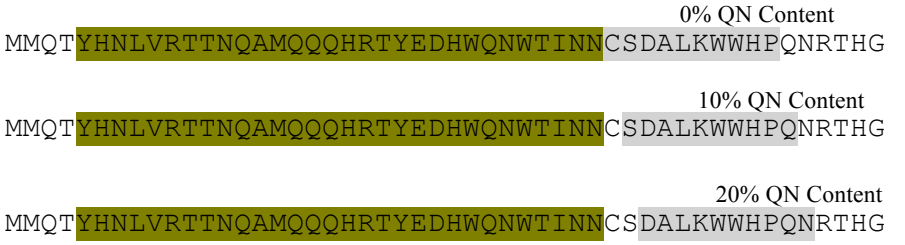


Figure 3.12. The GARRF Algorthm
A graphic representation demonstrating the GARRF algorithm on a random stretch of genome. Grey areas represent 10-residue windows that are being analyzed for QN content. Green areas are sequences flagged as QN rich

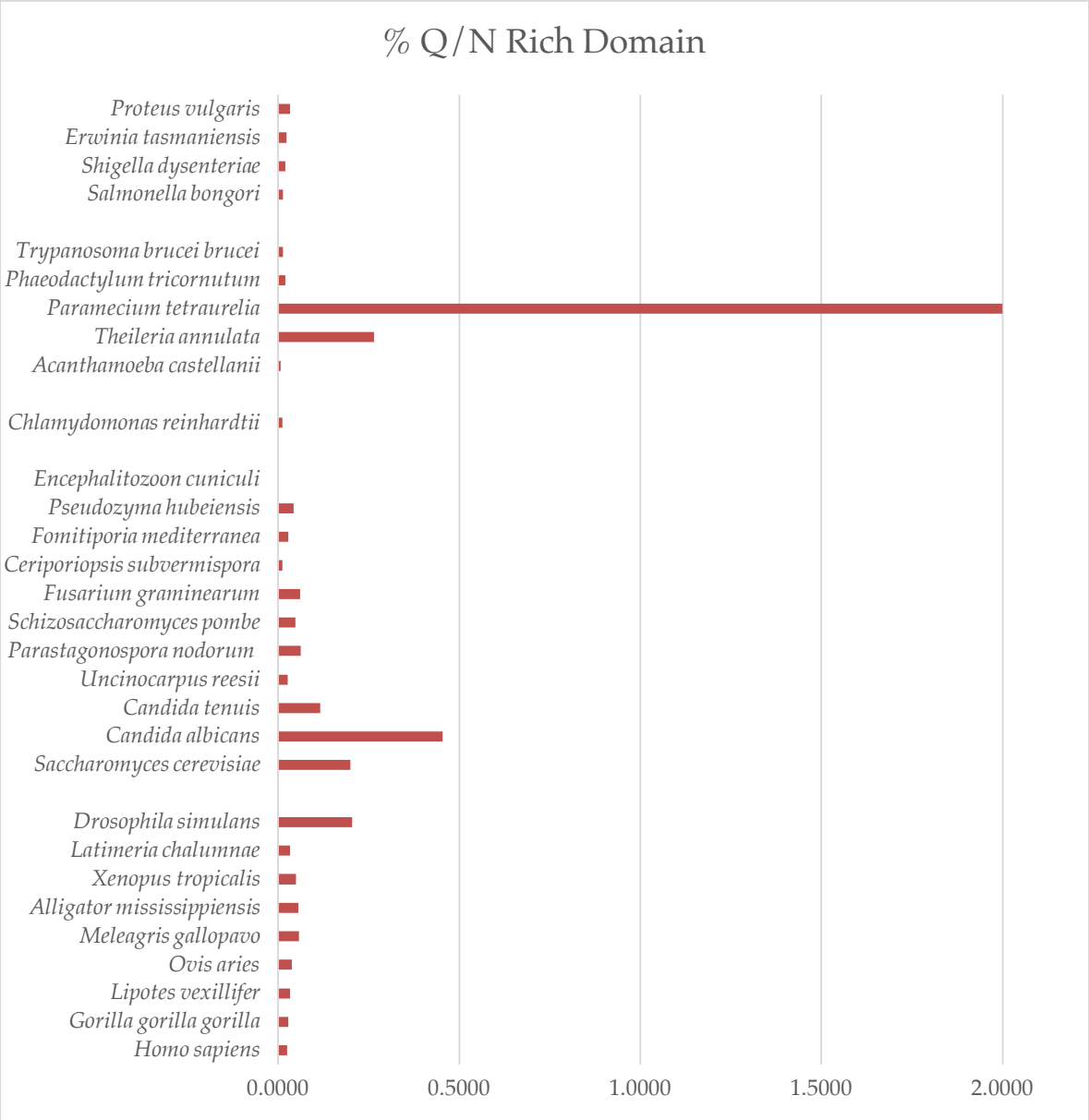


Figure 3.13 The percent of each genome that is Q/N rich
The GARRF algorithm was used to calculate the Q/N rich domain content of 30 species. The algorithm returns all domains 40 residues or longer, that have Q/N content greater than 30%. The total length of these domains was divided by the total length of the proteome to calculate the Q/N rich domain content. Q/N rich domains are most prevalent in single celled eukaryotes and the fruit fly.

Conclusions

BiFC Prion Assay

Initially this researcher didn't want to include this project in his dissertation. The assay, as it stands now, is not a useful experimental tool. However, after discussions with my committee, this researcher has come to realize that this line of investigation produced several pieces of information that are critical to the development of a BiFC assay and that shed some new light on the nature of Ure2p amyloid fibers.

First, the BiFC assay is able to detect prion aggregation. When yeast strains carrying BiFC fusion proteins are cultured in galactose media, a sub-set of the culture develops a fluorescent strong enough to identify via FACS analysis. This size and intensity of the sub-set of cells does not correlate to differences in expression efficiency (see the western blot in Figure 3.3), indicating that this is not just a function of the concentration of BiFC fusion proteins. This sub-set of fluorescent cells is enriched under conditions that are selective for [URE3]. When sorted out via FACS, the fluorescent cells consistently show aggregation under an epifluorescent microscope.

Only 17% of the cells that showed increased fluorescence in fluorescence activated cell sorting formed [URE3] cells in a loss-of-function yeast genetics assay. This is most likely due to the increased sensitivity of a BiFC based assay. Yeast genetics assays detect the loss-of-function of the aggregated protein. These assays only return a positive result if a majority of the prion protein is incorporated into amyloids, and if the amyloid is stably propagated over multiple generations. Thus, the BiFC assay is capable of detecting weaker and less stable strains of [URE3].

The presence of the BIFC fusion proteins does not dramatically affect the rate of [URE3] formation, as measured by the loss-of-function assay, indicating that fiber formation is not being driven by VN/VC interactions.

Structural Observations

Two strains were generated that were meant to serve as controls (strains 2 and 4). These had their VN and VC moieties on opposite ends of the prion domain. Neither of the strains behaved as expected. Both strains generated a clearly defined second population of higher fluorescence intensity when cultured in galactose media. The fluorescent population was enriched when cultured under conditions selective for the prion state for both strains. This is surprising as at least one model for the structure of the Ure2 amyloid fiber places the ends of the fiber out of reach of one another [224]. This implies that a significant portion of our VN/VC interactions may be from inter-fiber interactions.

Strains 1 and 3 show significantly different ability to form fluorophores, despite having their BiFC moieties located on the same side of the amyloid fiber. Likewise, strains 2 and 4 have significantly different fluorescent sub-populations despite having their venus moieties on the same side of the amyloid fiber. One similarity between the two most efficient strains is that the C-terminal end of the VN moiety is free to interact with the other BiFC fusion protein; this may be a requirements of efficient fluorophore formation.

Plasmid Stability

Unfortunately, the plasmid vector-based system used to deliver the BiFC constructs does not propagate consistently. Plasmid instability was exacerbated by BiFC construct expression, indicating that the presence of Ure2p aggregates interferes with plasmid segregation, possibly by direct physical interaction with the plasmid segregation mechanism. However, even in selective,

glucose media approximately 80% of the cells lost their plasmids over the course of a 48-hour culture. These results indicate that these vectors are unsuitable for use in a BiFC assay.

Future Work

This paper demonstrates that a BiFC assay is capable of detecting the presence of amyloid aggregation. The next step is to create a BiFC yeast strain that fulfills two requirements: first, it should express the Ure2 prion-forming domain at levels that are as close to bioequivalent as possible. Overexpression of the prion-forming domain increases the rate of prion formation and de-stabilizes the prion state [219]. The Ure2 prion fusion proteins were expressed from a high-copy plasmid under the control of an active promoter (GAL1). This was an appropriate approach for a proof of concept experiment, as we wanted to maximize that chances that we observe a fluorescence signal. Future work will focus on reducing the cellular concentration of Ure2 prion-forming domain while maintaining a strong signal.

Second, the fusion proteins should be expressed endogenously. Expressing the BiFC fusion proteins reduced the efficiency of plasmid propagation. Endogenous expression will side-step any issues with the aggregates disrupting plasmid segregation.

This researcher recommends the following approach. Constructs C and D should be transformed into the yeast genome under the control of the GAL1 promoter. A series of experiments could be performed where the BiFC constructs are transiently expressed for progressively longer periods of time to see how much fusion protein expression is required to detect prion aggregation without inducing it. It is worth mentioning that 2μ-plasmids are fairly high copy. Switching to a single copy of the gene will substantially reduce the level of expression. It is possible that switching to an endogenous promoter will reduce the expression level to a point where it does not spontaneously induce prion formation.

Bioinformatics

This researcher has always assumed that *S. cerevisiae* was an unusually Q/N rich species. This turned out to be true, but not dramatically so. On average, genomes were 0.14% Q/N-rich, only slightly below *S. cerevisiea's* 0.20%. There are a number of species that carry more Q/N-rich domains than *S. cerevisiea*, including fruit flies (0.21%), other fungi (*C. albicans*, 0.455%) and paramecium (1.99%). The species with the highest Q/N content seems to be clustered into several clades, a finding that is consistent with the hypothesis that these domains are evolutionarily optimized to serve some function. Q/N-content seems to be centered around single-celled eukaryotes, and the fruit fly. Unfortunately, the species themselves offer little insight as to what this function might be.

BIBLIOGRAPHY

1. True, H.L. and S.L. Lindquist, *A yeast prion provides a mechanism for genetic variation and phenotypic diversity.* Nature, 2000. **407**(6803): p. 477-83.
2. Halfmann, R., et al., *Prions are a common mechanism for phenotypic inheritance in wild yeasts.* Nature, 2012. **482**(7385): p. 363-8.
3. Halfmann, R. and S. Lindquist, *Epigenetics in the extreme: prions and the inheritance of environmentally acquired traits.* Science, 2010. **330**(6004): p. 629-32.
4. Nakayashiki, T., et al., *Yeast prions [URE3] and [PSI+] are diseases.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10575-80.
5. Wickner, R.B., et al., *The yeast prions [PSI+] and [URE3] are molecular degenerative diseases.* Prion, 2011. **5**(4): p. 258-62.
6. Paul, K.R. and E.D. Ross, *Controlling the prion propensity of glutamine/asparagine-rich proteins.* Prion, 2015. **9**(5): p. 347-54.
7. Tartaglia, G.G., et al., *Life on the edge: a link between gene expression levels and aggregation rates of human proteins.* Trends Biochem Sci, 2007. **32**(5): p. 204-6.
8. Vitrenko, Y.A., et al., *Propagation of the [PIN+] prion by fragments of Rnq1 fused to GFP.* Curr Genet, 2007. **51**(5): p. 309-19.
9. Zhou, P., I.L. Derkatch, and S.W. Liebman, *The relationship between visible intracellular aggregates that appear after overexpression of Sup35 and the yeast prion-like elements [PSI(+)] and [PIN(+)].* Mol Microbiol, 2001. **39**(1): p. 37-46.
10. Fernandez-Bellot, E., E. Guillemet, and C. Cullin, *The yeast prion [URE3] can be greatly induced by a functional mutated URE2 allele.* EMBO J, 2000. **19**(13): p. 3215-22.
11. Derkatch, I.L., et al., *Prions affect the appearance of other prions: the story of [PIN(+)].* Cell, 2001. **106**(2): p. 171-82.
12. Kawai-Noma, S., et al., *Dynamics of yeast prion aggregates in single living cells.* Genes Cells, 2006. **11**(9): p. 1085-96.
13. Masison, D.C., M.L. Maddelein, and R.B. Wickner, *The prion model for [URE3] of yeast: spontaneous generation and requirements for propagation.* Proc Natl Acad Sci U S A, 1997. **94**(23): p. 12503-8.
14. Baxa, U., et al., *Mechanism of inactivation on prion conversion of the Saccharomyces cerevisiae Ure2 protein.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5253-60.
15. Yu, Y., et al., *Flexibility of the Ure2 prion domain is important for amyloid fibril formation.* Biochem J, 2011. **434**(1): p. 143-51.
16. Moerner, W.E., et al., *Optical methods for exploring dynamics of single copies of green fluorescent protein.* Cytometry, 1999. **36**(3): p. 232-8.
17. Edskes, H.K., V.T. Gray, and R.B. Wickner, *The [URE3] prion is an aggregated form of Ure2p that can be cured by overexpression of Ure2p fragments.* Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1498-503.
18. Michelitsch, M.D. and J.S. Weissman, *A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 11910-5.
19. Sherman, F., *Getting started with yeast.* Methods Enzymol, 1991. **194**: p. 3-21.

20. Ranson, N., et al., *Insights into the architecture of the Ure2p yeast protein assemblies from helical twisted fibrils.* Protein Sci, 2006. **15**(11): p. 2481-7.
21. Shewmaker, F., et al., *Amyloids of shuffled prion domains that form prions have a parallel in-register beta-sheet structure.* Biochemistry, 2008. **47**(13): p. 4000-7.
22. Wickner, R.B., *[URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae.* Science, 1994. **264**(5158): p. 566-9.
23. Kodama, Y. and C.D. Hu, *Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives.* Biotechniques, 2012. **53**(5): p. 285-98.
24. Futcher, A.B. and B.S. Cox, *Copy number and the stability of 2-micron circle-based artificial plasmids of Saccharomyces cerevisiae.* J Bacteriol, 1984. **157**(1): p. 283-90.
25. Ross, E.D., et al., *Primary sequence independence for prion formation.* Proc Natl Acad Sci U S A, 2005. **102**(36): p. 12825-30.
26. Kajava, A.V., et al., *A model for Ure2p prion filaments and other amyloids: the parallel superpleated beta-structure.* Proc Natl Acad Sci U S A, 2004. **101**(21): p. 7885-90.

CHAPTER 4: CONCLUSIONS

Q/N-rich yeast prion-forming domains sit at the edge of the phase transition between structure and disorder. Slight shifts in the composition of a yeast prion-forming domain convert it to a permanently disordered state or a permanently ordered state.

This fact is highlighted by our experiments on the effects of composition changes on the prion propensity of Sup35. The prion-forming domain of Sup35 is 114 residues long, and 20.2% are prion-inducing hydrophobic residues (F, I, V, Y, M, W). A shift to 24.2% hydrophobic content converts the prion-forming domain to a permanently ordered region. Indeed, the insertion of as few as two hydrophobic residues results in a several orders-of-magnitude increase in prion formation, and significant acceleration of in vitro amyloid formation Conversely, eliminating the five tyrosines found in the first 40 residues of Sup35 almost completely eliminates the ability to aggregate. These findings corroborate the predictions made by PAPA, a prion propensity prediction algorithm developed in our lab.

It has been suggested that prion-forming domains may be an example of the "life on the edge" hypothesis put forward by Tartaglia et al. Tartaglia argues that although protein aggregation is evolutionarily disfavored, once a protein has evolved such that it is soluble within the narrow range of normal physiological conditions, there is no selective benefit to further reduce the aggregation propensity of a protein. This explains why most proteins are induced to aggregate by slight changes to the cellular environment. Both protein structure and expression levels have evolved to avoid aggregation, but they do so with very little margin of safety.

As appealing as the "life on the edge" hypothesis is, I believe that it does not apply to the yeast prions for a couple of reasons. First, it assumes that most point mutations destabilize

proteins. For most of the proteome this is true: point mutations tend to disrupt the delicately balanced forces that govern protein structure, disordering regions, rendering them prone to aggregation. This is demonstrated by the numerous point mutations that give rise to amyloid associated diseases [225]. However, yeast prion-forming domains are already intrinsically disordered, so mutations will not disrupt structure. Furthermore, we know how each possible point mutation will affect these Q/N-rich domains. Specifically, Toombs et al. used a mutagenic screen to test the prion propensity of each amino acid in the context of a scrambled copy of the Sup35 prion-forming domain [121]. They found that 12 of 20 residues either were approximately neutral, or decreased the likelihood of prion aggregation. So most point mutations do not actually increase the chances that a domain will behave as a prion. Furthermore, this theory assumes that a given domain only exerts selective forces on the cell when it is actively aggregating. Yeast prion domains are unique in that they require an ensemble of chaperone proteins to aggregate and propagate. Indeed, members of the HSP70 bind to Sup35p in its non-amyloid form and prevent prion formation in an ATP-dependent manner. So the cell is constantly spending energy to keep its many prion and prion-like domains in check. If they don't serve any function in the cell, one would expect them to be culled from the population by natural selection.

There are a number of other possible reasons why yeast carry prion-forming domains, in spite of their deleterious effects. They could be random genetic drift that occasionally results in the accumulation of QN-rich domains. Another possibility is that these are polyglutamine expansions undergoing entropic breakdown. In order to better explore these hypotheses we used the GARRF algorithm to search through the genomes of a diverse set of genomes. We found that Q/N-rich species are loosely clustered into several clades, single-cell eukaryotes and drosophila. One possible explanation for these findings is that these species are more prone to poly-

glutamine expansions that other species, and thus have more polyglutamine entropic decomposition occurring. However, there is no evidence that these species suffer from excessive poly-glutamine expansion.

An Alternate Theory Explaining the Existence of Prion-forming Domains

Another hypothesis is that these species have evolved poly-QN domains as functional amyloids, evolved to serve a cellular purpose. In this model, prion-forming domains are optimized to aggregate, but not necessarily form prions. It is possible that prion-forming domains are evolutionarily optimized to aggregate in response to stress in a dynamic, reversible manner, not unlike stress granules. When the stress ends, the aggregation ceases and the cell returns to normal. This is different from prions, which are caused by stable, transmissible, self-catalyzing alternative protein structures, passed from mother to daughter.

This aggregation could serve as a mechanism for the cell to detect and respond to stresses that cause protein misfolding. Prion-forming domains are prone to aggregation. Indeed, in vitro they spontaneously aggregate into amyloid fibers. Presumably, in vivo prion nucleation is constantly suppressed by chaperone activity. When the cell's ability to mediate aggregation is disrupted, either by increased prion nucleation or inhibition of chaperone activity, prion proteins are sequestered into amyloid fibers.

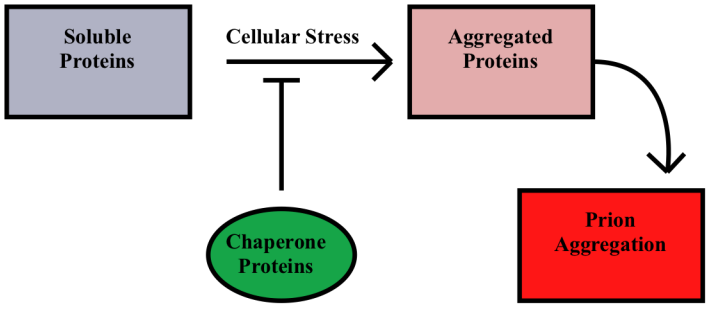


Figure 4.1 An alternate theory of prion function. **1.** Prion-forming domains are kept soluble by chaperone proteins. **2.** Cellular stress overwhelms chaperone activity, resulting in prion-forming domain aggregation. **3.** After stress is removed prion-forming domains return to their soluble state. **4**. Prolonged stress can result in an aggregant that the cell can not cure; i.e. a prion.

The structural similarities shared by all prion proteins may shed some light on how they operate. Prion proteins have a disordered prion-forming region linked to an ordered domain. These ordered domains serve a number of functions, but they all operate as protein expression regulators, mediating either transcription or translation of a diverse array of proteins [226, 227]. So when amyloids form, they sequester a subset of protein regulation domains, altering the proteome. When stress is removed from the cell, the chaperone machinery would disaggregate amyloid aggregates, returning the proteome to its unstressed state.

Prion-forming domains present a number of advantages as protein misfolding detectors. Many types of stress cause protein misfolding including heat, cold, starvation, redox extremes, and a host of xenobiotic agents. Creating cellular detectors for every possible source of misfolding may not even be possible. PFDs may provide an extremely generalized protein misfolding response.

Work in the Ross laboratory demonstrated that the composition of prion-forming domains is finely tuned to allow the prion-forming domain to aggregate at a rate just slow enough that the chaperone mechanism suppresses amyloid growth. Even a single-residue mutations can dramatically alter the rate of prion formation, creating domains that are either constantly in the amyloid state or that almost never nucleate. In a sense, prion domains are like canaries in a coal mine, poised to aggregate at the slightest provocation. This aggregation "warns" the cell of impending cell-wide aggregation that could threaten other non-prion proteins, so that they can initiate a cellular response to mitigate further protein aggregation and cell damage.

If prion-forming domains did not evolve to form prion, why do prions occur? Prions may be a rare event where aggregated prion domains overwhelm the cell's ability to clean house, creating semi-permanent aggregates. This may occur as the result of prolonged exposure to

stress, allowing aggregates to grow large enough that they cannot be cleared, or by the occurrence of a particularly rabid variant that grows faster than the cell can clear.

Is there any supporting data for this theory? Song et al. demonstrated that growth on $MgCl^2$-induced Sup35p aggregation in vivo in a dose-dependent and reversible manner [228]. Specifically they show that when exposed to $MgCl^2$, cells adopt a weak, unstable prion phenotype (they grow light pink on the ½ YPD assay). They also demonstrate that this phenotype correlates to the in vivo aggregation of Sup35p, although the aggregates are not as large as those caused by the prion state.

The authors don't seem to appreciate the significance of their finding, claiming that these results "have provided some evidence to confirm the hypothesis that [PSI+] represents a mechanism for evolvability at the cellular and protein levels". Here they are referring to Susan Lindquist's theory that prions are genetic capacitors that allow for increased phenotypic diversity in response to stress.

Prion-forming domain aggregation is regulated by chaperone proteins in a complicated, ATP-dependent manner, hinting that it is a native cellular mechanism. Indeed, activation of the heat-shock response is strikingly similar to what we know about how prion aggregation initiates. In yeast, the heat-shock proteins from the Hsp70 and Hsp90 family form a complex with the heat-shock transcription factor Hsf1. As heat-shock proteins are titrated away from the complex to combat misfolding events, Hsf1 is released to initiate heat-shock response [229]. Similarly, an Hsp70:Hsp40 complex has been shown to suppress amyloid formation in vivo in yeast. It has been proposed that titrating away these same chaperones initiates prion formation [86].

Why has this theory gone unexplored? Yeast prions were first discovered in the 1990's during an outbreak of New Variant Creutzfeld-jakob disease in Great Britain. At that time, the

most interesting aspect of prion-forming domains is that they could behave in a manner resembling mammalian prion diseases.

Also, the way we study yeast prions limits our understanding of the phenomena. Many yeast genetics studies require a prion-infected strain of yeast. Isolating a prion strain involves screening through variants to find one that is both strong enough that it is easily assayed for, and stable enough that it doesn't lose its phenotype in the middle of an experiment. Unfortunately, this process creates a kind of experimental tautology. Screening out all examples of transient protein aggregation before you start your experiment precludes one from making important discoveries involving transient protein aggregation.

Most yeast genetics techniques involve plating a single yeast cell to selective media, and waiting for it to grow into a colony. A yeast colony grows to approximately 1-10 million cells before it is visible to the naked eye. So as long as we rely on yeast genetics assays, we will have to study strains that are stable enough to propagate for a minimum of approximately 20 consecutive cell divisions.

Our work on a BiFC assay is a good start at opening up the yeast prion field to alternative hypotheses. We have demonstrated that amyloid fibers can be detected using BiFC reporter. Furthermore, we established a roadmap to further develop the assay to the point that it is a useful laboratory tool. Taken together, these findings advance our field in incremental but vital steps.

BIBLIOGRAPHY

1. Wetzel R. Mutations and off-pathway aggregation of proteins. Trends Biotechnol. 1994;12(5):193-8.
2. Toombs JA, McCarty BR, Ross ED. Compositional determinants of prion formation in yeast. Mol Cell Biol. 2010;30(1):319-32.
3. Harbi D, Harrison PM. Classifying prion and prion-like phenomena. Prion. 2014;8(2).
4. Harbi D, Harrison PM. Interaction networks of prion, prionogenic and prion-like proteins in budding yeast, and their role in gene regulation. PLoS One. 2014;9(6):e100615.
5. Song Y, Lan W, Wu X, He J, Li H, Ben S, et al. Quantitative effects of magnesium chloride stress on aggregation of Sup35p in [psi-] yeast cells. Protein Pept Lett. 2010;17(12):1489-94.
6. Richter K, Haslbeck M, Buchner J. The heat shock response: life on the verge of death. Mol Cell. 2010;40(2):253-66.
7. Gonzalez Nelson AC, Ross ED. Interactions between non-identical prion proteins. Semin Cell Dev Biol. 2011;22(5):437-43.

ADDENDUM 1: INTERACTIONS BETWEEN NON-IDENTICAL PRION PROTEINS

Adapted from:

INTERACTIONS BETWEEN NON-IDENTICAL PRION PROTEINS

Aaron C. Gonzalez Nelson and Eric D. Ross

Introduction

Prion formation involves the conversion of soluble proteins into an infectious amyloid form. This process is highly specific, with prion aggregates templating the conversion of identical proteins. However, in some cases non-identical prion proteins can interact to promote or inhibit prion formation or propagation. These interactions affect both the efficiency with which prion diseases are transmitted across species and the normal physiology of yeast prion formation and propagation. Here we examine two types of heterologous prion interactions – interactions between related proteins from different species (the species barrier) and interactions between unrelated prion proteins within a single species. Interestingly, although very subtle changes in protein sequence can significantly reduce or eliminate cross-species prion transmission, in *Saccharomyces cerevisiae* completely unrelated prion proteins can interact to affect prion formation and propagation.

Background

Prions are protein-based infections. The prion concept was first proposed to explain the unusual ability of the sheep disease scrapie to transmit without any apparent nucleic acid

component [230]. Since then, prions have been implicated in a number of mammalian neurodegenerative diseases, including Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy in cattle, and chronic wasting disease in deer and elk [231]. All mammalian prion diseases are caused by a structural conversion in the cellular protein PrP. In its native state, PrP is an α-helix-rich surface protein that is attached to the exterior of the cell via a glycocylphosphatidylinositol anchor. Prion formation results from conversion of PrP into a self-propagating β-sheet-rich amyloid-like form.

Interestingly, fungi have been found to carry a number of amyloid-based prions. The most highly studied of these, [URE3], [PSI+] and [PIN+], are the prion forms of the *Saccharomyces cerevisiae* proteins Ure2, Sup35 and Rnq1, respectively [2, 50, 170]. Each protein contains a glutamine/asparagine (Q/N) rich prion domain that is thought to convert from an intrinsically disordered form to a β-sheet-rich amyloid form upon prion formation [232-234]. In the prion state, these proteins are sequestered into amyloid deposits, causing a change in protein activity. The prion state can be stably passed from mother to daughter cell during mitosis, allowing the prions to act as protein-based genetic elements [235]. Under some circumstances, prions may serve a beneficial regulatory function [236]. In yeast, [PSI+] has been proposed to provide an epigenetic mechanism to survive cellular stress, allowing cells to temporarily adapt to adverse conditions [142, 237, 238]; however, the failure to identify [PSI+] in wild yeast strains has been used to argue that [PSI+] is not used as a regulatory element in nature [131].

Prion propagation is generally highly sequence-specific. Prion aggregates cause the structural conversion of homologous prion proteins. However, in some circumstances heterologous prion proteins can positively or negatively interact. The phrase “heterologous prion proteins” has been used to describe two different types of interactions – interactions between the

same protein from two different species (the species barrier), and interactions between unrelated proteins within a species. Both involve the same phenomenon – interactions between proteins with non-identical amino acid sequences. Such interactions have significant physiological implications. They govern the efficiency with which prions are spread between species. Additionally, because yeast contain multiple unrelated prion proteins, interactions between these proteins can both positively and negatively affect prion formation and propagation. Thus, understanding how prions interact with one another will yield insight into the infectivity of mammalian prion diseases and the normal physiology of yeast prion formation and propagation. Furthermore, similar interactions between unrelated amyloid proteins may affect the onset and progression of various amyloid diseases, so studies of interactions between heterologous prion proteins may provide insight into non-prion amyloid diseases. Here, we examine such interactions between heterologous prion proteins.

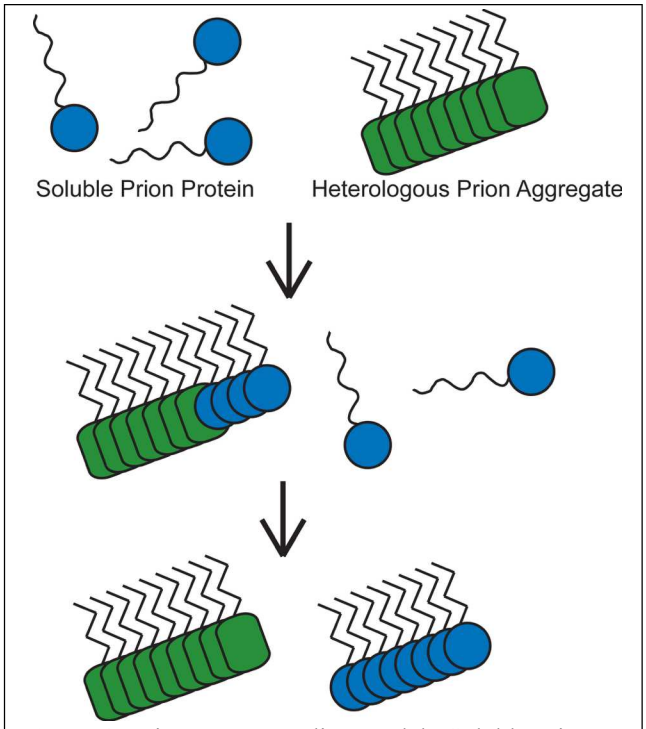


Figure 1. Direct cross-seeding model. Soluble prion proteins (blue) directly interact with exposed β-edges on heterologous prion aggregates (green), converting the soluble protein to the prion form. For [PSI+]/[PIN+] cross-seeding, this interaction appears to be transient, with [PSI+] and [PIN+] forming distinct prion aggregates.

The Basis for Heterologous Prion Interactions

Heterologous prions could influence each other through direct interaction (Figure 1), or by competing for, or changing expression levels of, cellular factors that positively or negatively influence prion formation and propagation (Figure 2). These mechanisms are not mutually exclusive. In fact, although positive interactions between heterologous prions seem to be driven primarily by direct interactions, there is some evidence supporting both models.

Direct interactions between heterologous prion proteins

The structure of amyloid fibrils provides insight into both the basis for specific recruitment of homologous proteins into prion fibrils, and the possible mechanism for recruitment of heterologous proteins. Amyloid is characterized by a cross-β structure, in which the β-strands donated from each protein subunit run perpendicular to the fiber axis. The precise three-dimensional structure has not been solved for any prion aggregate. For PrP, EPR and solid state NMR suggest that the C-terminus of PrP adopts an in-register parallel β-sheet structure within amyloid fibrils [42, 239], although other structures have been proposed [41]. Likewise, solid state NMR suggests that Ure2, Rnq1 and Sup35 each adopt an in-register parallel β-sheet structure in the amyloid form [35, 240, 241], although for Sup35 a β-helix structure has also been proposed [62].

The cross-β structure (either β-helix or in-register parallel β-sheet) nicely explains the ability of fibers to template the structural conversion of homologous proteins, as well as the specificity of this activity. The ends of fibers contain exposed β-edges, which serve as recruitment sites for soluble protein. The ability of a protein to bind to these ends is dependent on the compatibility of the protein's sequence with the existing β-structure, creating specificity.

The proposed fiber structure also provides insight into the possible basis for heterologous cross-seeding. Because β-sheets are stabilized by hydrogen bonds between the backbones of adjacent β-strands, a perfect sequence match is not required between the aggregated and heterologous soluble protein; it may just require structural compatibility of the heterologous protein with a specific exposed amyloidogenic stretch. In addition to backbone interactions, this compatibility could involve various side-chain interactions that have been proposed to stabilize amyloid fibrils. These include hydrophobic interactions [242], charge-charge interactions [243], formation of polar zippers by Q/N residues [244], or interdigitation of side-chains to form a steric zipper [59].

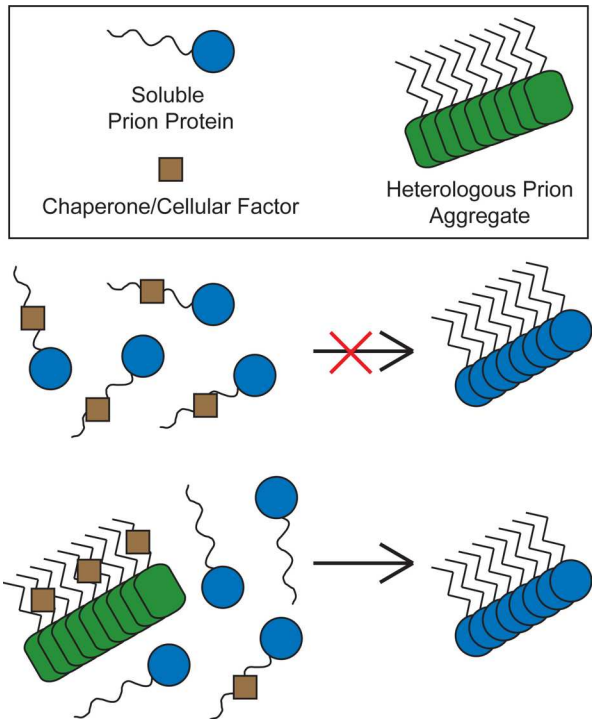


Figure 2. Titration model for indirect interactions between prion proteins. Cellular factors (brown squares) bind to prion proteins (blue), preventing prion formation. The presence of heterologous prion aggregates (green) titrate away these cellular factors, facilitating prion formation. This model was originally proposed to explain the ability of the yeast prion [PIN+] to promote [PSI+] formation [1, 2].

Indirect interactions between heterologous prions

There are three basic steps required for a protein to act as an amyloid-based prion [[200]]. First, some number of proteins needs to undergo structural conversion and aggregation to generate a prion seed or nucleus. Second, this seed needs to recruit additional soluble proteins and convert them to the misfolded form, thereby growing the prion aggregate. Finally, the prion aggregates need to be fragmented to generate new seeds. In yeast, prion maintenance requires

new seeds to be generated at a sufficient rate to compensate for dilution by cell division. In mammals, generation of new seeds is likely required to offset clearance of aggregates and to allow for spread of disease.

Each of these three steps is likely influenced by cellular factors such as chaperones. These factors are best understood in yeast (for review, see [245]). Hsp104, a chaperone involved in fragmentation of prion aggregates to generate new seeds [199-201], is necessary for efficient propagation of all known amyloid-based yeast prions [246]. Likewise, levels of Hsp40s and Hsp70s can influence the efficiency of prion formation and propagation (for review, see [245]). Prions could affect the formation and stability of heterologous prions either by competing for interacting cellular factors (Figure 2) or by changing expression levels of these factors.

Species Barrier

Mammalian prions are generally transmitted less efficiently between different species than within species [247]. Likewise, in yeast, prions are transmitted more efficiently between cells expressing prion proteins derived from the same species of yeast than between cells expressing prion proteins derived from different species [204, 248-250]. This phenomenon is called the species barrier.

Mammalian species barrier

In mammals, cross-species inoculation of prion diseases results in a lower attack rate and requires a longer incubation period than same-species inoculation [247]. However, after multiple passages through the same species, the heterologous prion strain adapts to its host, and the species barrier is eliminated [31].

Because prion diseases affect mammalian species that are distantly related to one another (i.e. mice and humans), the species barrier could simply be a function of physiological

differences between the host species. Alternatively, the species barrier could result from differences in PrP between the donor and the recipient. Transgenic mouse studies strongly argue in favor of the second explanation. Transgenic mice expressing heterologous PrP do not show the species barriers that are typical for mice; instead, the species barrier is determined by the source of the PrP gene [251].

However, the species barrier is not mediated solely by primary sequence; it is also dependent on the specific prion variant being transmitted. A single prion protein can adopt different prion variants, each with different biological characteristics [31]. Variants (also called strains) are prions that are sequentially identical but structurally different [67, 252, 253]. In mammals, different prion variants have widely varying incubation times, attack rates, and patterns of brain lesions. In yeast, prion variants are distinguished by how much of the prion protein is sequestered and how consistently the prion propagates during mitosis. Transmission studies in mice confirm that the extent of the species barrier is prion variant-specific [254], demonstrating that the extent of the species barrier is not solely a function of differences in primary sequence. This is consistent with the direct cross-seeding model, as the efficiency of heterologous interactions should be governed not by the sequence similarity between two proteins, but by the structural compatibility between the prion aggregate and the heterologous protein.

Mammalian PrP is fairly highly conserved, with a sequence identity of 90% or better between most species. How can these seemingly minor differences, in some cases, completely abrogate inter-species transmission? For any given PrP sequence, an ensemble of variants will be favorable. Single mutations can both positively or negatively influence prion propensity [255]; thus, small changes can likely also affect the constellation of conformations available to a given

protein, and thus affect the compatibility of a soluble protein with the structure found at a fiber end.

Yeast Species Barriers

Studies of the mammalian species barrier have been limited by intrinsic challenges of mammalian systems. Notably, refolding of purified recombinant PrP into amyloid fibrils produces material of only minimal infectivity; co-factors are required for efficient generation of infectivity [25]. The inability to efficiently generate infectivity from purified protein makes biophysical studies difficult. Furthermore, the correlation between in vivo and in vitro PrP species barrier experiments remains unclear [256]. Yeast prions do not have these limitations. Amyloid from recombinant yeast prion proteins is highly infectious [67, 257], and the species barrier can be replicated in vitro [204]; this,

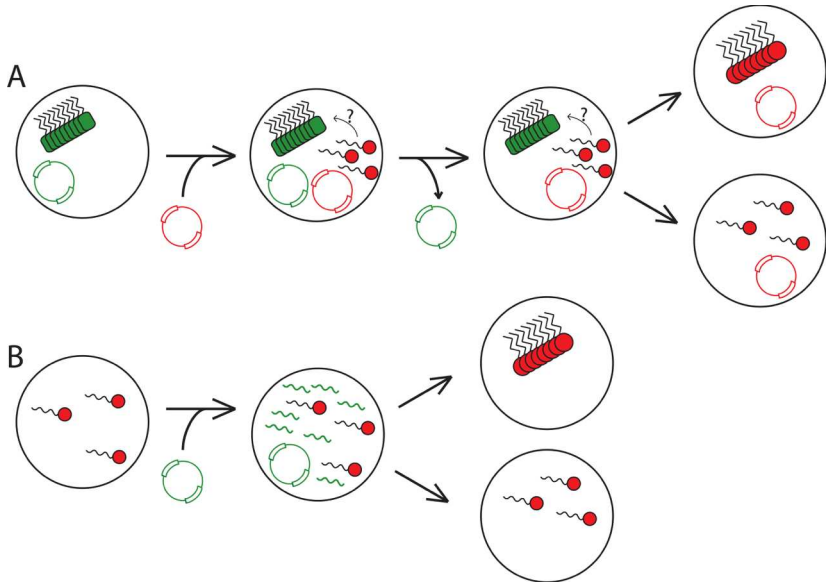


Figure 3. Methods to investigate heterologous prion interactions. (A) Plasmid shuffling. A plasmid expressing a heterologous version of Sup35 (red) is introduced into a [PSI+] strain expressing the sole copy of SUP35 from a plasmid (green). After selection for loss of the original Sup35-expressing plasmid, the cells are assayed for [PSI+]. (B) Induction experiments. An inducible plasmid expressing a heterologous prion domain (green) is introduced into a cell carrying a genomic copy of the endogenous full-length prion protein (red). After transient overexpression of the heterologous prion domain, cells are assayed for prion formation.

combined with the rapid growth rate and ease of genetic manipulation of yeast, has made yeast prions a powerful model for exploring the basis for the species barrier (for a review, see [258]).

The species barrier has been most extensively studied for Sup35. The prion domain from *S. cerevisiae* contains an N-terminal Q/N rich region (residues 1-40), followed by an oligopeptide repeat domain (residues 41-114) containing five and a half imperfect repeats of a nine-amino acid segment [191, 259]. The prion domains of Sup35 from various fungal species have been investigated [204, 248-250] . Sup35 from each species is compositionally similar. All have high Q/N content, relatively few charged residues and a repeat domain. Sequentially there is a great deal of variation within the set, with the most closely related species (S. paradoxus) having 97% sequence identity with *S. cerevisiae*, and the most distantly related having only about 20%.

Plasmid shuffling (Figure 3A) has been used to investigate the species barrier between *S. cerevisiae* and foreign Sup35s [204, 249, 250]. As with PrP, a species barrier was observed, even between some closely related Sup35s. Sup35 from *C. albicans*, *K. lactis* and *P. methanolica* can each form stable prions when expressed as the sole copy of Sup35 in *S. cerevisiae*; however, when plasmids expressing these heterologous versions of Sup35 were shuffled into [PSI+] cells expressing *S. cerevisiae* Sup35, the heterologous Sup35s were not efficiently converted to the prion state, demonstrating the presence of a species barrier [204, 250]. By contrast, Sup35 from the more closely related S. paradoxus and S. bayanus were occasionally able to cross this species barrier [249]. Interestingly, both *S. paradoxus* and *S. bayanus* Sup35 co-aggregate with *S. cerevisiae* Sup35, but this co-aggregation is not sufficient to bridge the species barrier [249]. This suggests that the species barrier is not solely limited by the ability of heterologous proteins

to co-aggregate; the heterologous protein must be compatible with, and therefore able to propagate, the existing prion structural variant.

Chimeric proteins have provided a powerful tool for identifying the regions responsible for the species barrier. One such chimera, in which residues 41-123 of *S. cerevisiae* Sup35 were replaced with the corresponding sequence from *C. albicans* [204, 260], can form different prion variants in vitro depending on the conditions used for aggregation [261]. Both in vitro and in vivo, this chimera can be seeded by either *S. cerevisiae* or *C. albicans* Sup35, but then maintains the species barrier specificity of the original source of infection [260, 261]. This suggests that the key region required to cross the species barrier is species specific.

A more systematic analysis of the minimal region required to cross the species barrier has likewise shown that no single region of Sup35 is solely responsible for mediating the species barrier. Chen et al. subdivided the prion domain of Sup35 into three parts [203]. Chimeric prion domains were created using different combinations of the three segments from different species. The ability of the chimeric proteins to overcome the species barrier was tested using plasmid shuffling. No single domain or residue dictated species specificity. Instead, different modules were important for overcoming different species barriers.

Sup35 contains three “amyloid stretches” – consensus hexapeptides that are found in many amyloid-forming proteins [3]. Interestingly, polymorphisms in each amyloid stretch had a significant effect on specific species barriers [203]. This suggests that identity within specific short, highly amyloidogenic segments may be critical for crossing certain species barriers. This might seem to conflict with recent studies examining the sequence basis for the yeast prion propensity. Although multiple algorithms have been designed to identify short stretches of high amyloid propensity, these algorithms are not effective at distinguishing between Q/N-rich

proteins with and without prion activity; instead, yeast prion domains are distinguished by the presence of large disordered domains of modest prion propensity [122, 172]. However, these results need not be contradictory. As discussed in Section 2.2, three distinct steps are required for a protein to act as a prion. For Sup35, a significantly larger fragment is required for efficient prion propagation than for addition onto pre-existing [PSI+] aggregates [190]. Likewise, it is reasonable that short segments could play a key role in nucleating cross-seeding, but not be sufficient to determine overall prion propensity.

Peptide arrays provide an alternative method to investigate the specific regions that mediate the species barrier. Tessier et al. created surface bound arrays of short (20-mer) segments of the Sup35 protein [205]. They incubated full-length fluorescently-labeled Sup35 with the array to identify domains that were capable of amyloid nucleation. For S. cerevisiae Sup35, 20-mers from the 9-39 region were capable of nucleating fiber formation. By contrast, *C. albicans* Sup35 was nucleated by residues in the 59-86 region. These results are consistent with analysis of the *S. cerevisiae/C. albicans* chimeric Sup35 protein, as this protein contains both the *S. cerevisiae* and *C. albicans* nucleation regions, and was able to be seeded by both *S. cerevisiae* and *C. albicans* Sup35 aggregates [260]. On the arrays, the chimera was able to interact with both recognition domains.

These experiments show that contact between discrete interaction domains is required to nucleate fiber formation. However, polymorphisms entirely outside of the prion domain can also influence the species barrier [203], highlighting the complex nature of the species barrier. Such mutations likely affect the prion protein structure, affecting compatibility with heterologous prion domains.

Although it has not been as extensively studied as Sup35, similar results have been seen for Ure2 [262]. Ure2 from a variety of *Saccharomyces* species can form prions when expressed in *S. cerevisiae*. Although the sequences are highly similar (>90%), a clear species barrier is seen in transmission experiments [262]. Furthermore, the species barrier seems to be variant-specific.

To date, we do not have precise three-dimensional structural data for any prion. We also lack a clear understanding of the pre-amyloid molten state that proteins adopt before being recruited to amyloid [263]. This makes it difficult to predict how specific polymorphisms affect prion transmissibility. However, we can draw some broad conclusions from this work. Crossing the species barrier clearly involves direct interactions between heterologous proteins. The species barrier is determined not just by the sequence identity between the proteins, but also by the structural compatibility between the heterologous protein and the growing fiber end. Specific nucleating regions appear key to crossing a given species barrier. Variants likely hinder or induce prion seeding by mediating the exposure of interaction domains or by shifting the portion of the prion domain within the amyloid core.

Interactions between unrelated prion proteins

[PIN+]

Growth on low concentrations of guanidine HCl "cures" [PSI+] [264] by inhibiting Hsp104 [197, 198], a chaperone required for [PSI+] propagation [72]. Because prion formation is thought to result from a random misfolding event, cells that have lost [PSI+] should be able to spontaneously reacquire it [50]. However, when [PSI+] cells are treated with guanidine, only a subset maintain the ability to efficiently reform [PSI+] [196]. These cells were termed [PIN+], for [PSI+] inducibility.

[PIN+] is a heritable, dominant, Hsp104-dependent non-Mendelian phenotype [196] that can be transmitted through cytoplasmic transfer [2]. Additionally, cells cured of [PIN+] can spontaneously reacquire it [265]. Based on these characteristics, Derkatch et al. hypothesized that [PIN+] may be a prion [265]. Contemporaneously, the yeast protein Rnq1 was found to form prion aggregates [170]. Rnq1 was determined to be the [PIN+] prion protein when it was shown that Rnq1 deletion causes [PIN+] loss and prevents cells from being infected with [PIN+] by cytoplasmic transfer [2]. To date, the Rnq1 protein has no known function other than to promote the formation of other prions.

Most research suggests that [PIN+] induces [PSI+] by direct cross-seeding between Rnq1 fibers and soluble Sup35. In vivo, when [PSI+] formation is induced by overexpression of the Sup35 prion domain, newly formed Sup35 aggregates co-localize with Rnq1 aggregates [266], and Sup35 aggregates isolated from such cells contain some Rnq1 [179]. Further supporting a direct cross-seeding model, fusing the prion domain of Sup35 to Rnq1 in a [PIN+] strain, and therefore increasing the collision frequency between Sup35 and Rnq1 aggregates, significantly increases [PSI+] formation [267]. In vitro, pre-formed Rnq1 aggregates seed Sup35 aggregation [266]. These findings suggest that Rnq1 aggregates are capable of directly templating Sup35 aggregation. However an additional role for other factors in mediating this process has not been excluded. Newly formed Sup35 aggregates observed upon overexpression in [PIN+] cells are often associated with actin patches [268], suggesting that the cytoskeleton may be involved in concentrating misfolded proteins, thereby mediating interactions between prions.

Although [PIN+] is required for [PSI+] formation, it is not required for [PSI+] maintenance [265]. The Sup35/Rnq1 co-localization observed upon [PSI+] induction decreases over time [266], and in established [PIN+][PSI+] strains, [PIN+] and [PSI+] do not form mixed

subparticles [269]. Furthermore, [PIN+] generally does not affect either the average size of [PSI+] subparticles [269] or the fraction of Sup35 aggregated in a [PSI+] cell [265]. This implies that [PIN+] can seed [PSI+] formation, but that established [PIN+] and [PSI+] remain structurally separate.

[PIN+]'s activity is relatively non-specific. Overexpression of a variety of Q/N-rich proteins can substitute for [PIN+], allowing Sup35 to form prions in the absence of [PIN+] [1, 2]. [PIN+] also promotes, but is not required for, [URE3] formation [270], and the presence of [URE3] or [PSI+] promotes [PIN+] formation [2].

Given that even small changes in a protein's sequence can be sufficient to create a species barrier, the ability of unrelated proteins to cross-seed might seem surprising. However, these two ideas are not incompatible. Self-seeding is a remarkably efficient process; even in an actively growing yeast cell, prion aggregates are able to recruit the majority of the homologous protein in the cell. By contrast, seeding of [PSI+] formation by [PIN+] is quite inefficient; even in a [PIN+] strain, efficient [PSI+] formation requires strong overexpression of the Sup35 prion domain. Therefore, species barrier and [PIN+] experiments are asking fundamentally different questions. Species barrier experiments ask whether related proteins can efficiently seed each other; [PIN+] experiments ask whether a prion can very rarely promote the formation of heterologous prions.

So how do unrelated proteins such as Rnq1 and Sup35 cross-seed? The exact sequence basis for [PIN+]'s activity is unclear. The proposed prion domain is large, spanning amino acids 153–405 [271], and contains multiple distinct prion determinants [272]. Mutations throughout this region can affect [PSI+] induction, and some mutations seem to have variant-specific effects [273]. A single [PIN+] variant can promote formation of multiple [PSI+] structural variants.

Therefore, [PIN+] does not seem to directly structurally template prion formation by Sup35, but instead facilitates de novo prion formation. This is a fundamentally different process than homologous aggregation. While prion aggregates provide a direct structural template for conversion of homologous proteins (or closely related proteins), [PIN+] likely just provides a nucleating surface for Sup35 through weak or transient interactions. It is not surprising that small sequence changes might prevent direct structural templating, but that nucleation of de novo prion formation by heterologous proteins could accommodate much larger sequence differences. Because of the high Q/N content and relative lack of compositional complexity of yeast prion domains, there are likely to be many short stretches of complementarity between most yeast prion proteins; such stretches may be sufficient for nucleating interactions.

Interestingly, it appears that the line between these two mechanisms is not absolute. A recent examination of the species barrier between *S. cerevisiae* and *P. methanolica* Sup35 suggests that for a very strong species barrier, crossing the species barrier may involve a mechanism similar to [PIN+]'s cross-seeding of [PSI+] [274]. The *S. cerevisiae* and *P. methanolica* Sup35 prion domains have only about 32% homology. When both are in the prion form in the same cell, they propagate independently and form separate sub-particles, like [PIN+] and [PSI+]; this suggests that any interactions between these prion domains is weak and/or transient [274]. Furthermore, in species barrier experiments, in the rare cases where this species barrier is bridged, multiple distinct prion variants were observed [274]. This suggests that the species barrier is bridged by facilitating de novo prion formation by the heterologous Sup35, not by direct structural templating.

Ure2 promiscuity

Scrambled versions of Ure2, in which the order of the amino acids in the prion domain is randomized, maintain the ability to form prions [168]. Interestingly, in prion induction experiments (Figure 3B), over-expression of these scrambled domains in yeast increases the frequency of [URE3] formation by wild-type Ure2 [186]. In vitro, amyloid seeds from scrambled prion domains efficiently seeded amyloid growth by wild-type Ure2. In vivo, aggregates of fluorescently labeled scrambled prion domains partially overlapped with aggregates of fluorescently labeled wild type Ure2, suggesting that the induction is mediated by a direct, but transient interaction between proteins, similar to [PIN+]. Overexpression of various compositionally similar fragments from other yeast proteins similarly induced [URE3] formation. However, there does appear to be some specificity to this activity; over-expression of wild-type or scrambled Sup35 prion domains did not stimulate [URE3] formation.

Although this effect is reminiscent of the ability of various Q/N-rich proteins to substitute for [PIN+] in facilitating [PSI+] formation, the efficiency of these effects is quite different. Rnq1 aggregates are at least 50-fold less efficient than Sup35 aggregates at seeding Sup35 aggregation [266]. By contrast, the scrambled Ure2 prion domains induced [URE3] at levels comparable to wild type prio n domain [186]. Therefore, further experiments are required to determine the basis for and physiological relevance of Ure2's unique promiscuity.

Antagonistic prion-prion interactions

Interestingly, not all interactions between prions are positive; some yeast prions antagonize one another. For example, [URE3] strains spontaneously form [PSI+] at a much lower rate than cells lacking [URE3], and vice versa [270, 275]. Additionally, the presence of

[URE3] lessens the severity of the [PSI+] phenotype, and vice versa [275]. A similar discovery was made regarding specific strains of [PSI+] and [PIN+]. Certain variants of [PIN+] destabilize some, but not all [PSI+] variants [276]. It is unclear whether this is the result of a direct or indirect interaction. Schwimmer and Masison found that [URE3] and [PSI+] have distinct effects on chaperone levels, and also have differential sensitivity to certain chaperones [275]. Therefore, they proposed that heterologous prions can affect each other by shifting the chaperone balance in the cell. However, it is also possible that a direct interaction between the heterologous prion aggregates causes prion destabilization. Therefore, while it is clear that prion aggregates have an ongoing relationship that extends beyond the initial cross seeding event, the exact basis for this effect is still unknown.

Conclusions

On their surface, the species barrier and the [PIN+]/[PSI+] interaction seem like completely different phenomena. The [PIN+]/[PSI+] interaction occurs between sequentially unrelated proteins, while the species barrier can appear absolute, even with relatively high sequence identity. However, the mechanism underlying these phenomena seems to be similar. Direct physical interaction between heterologous prion domains are thought to drive both processes. To have this direct physical interaction, the exposed beta edge of an amyloid must be structurally compatible with its interacting heterologous protein. This likely explains how small sequence changes can significantly reduce the efficiency of cross-seeding, yet unrelated proteins can, under rare circumstances, transiently interact to seed prion formation.

Although it is clear that a direct physical interaction is key to mediating these effects, the details of these interactions and the role of other cellular factors is still not fully understood.

Furthermore, it is unclear why Ure2 interacts so efficiently with compositionally similar proteins. Addressing these issues will be important for understanding mammalian cross-species prion transmission, and for understanding the normal physiology of yeast prions. Additionally, such studies may provide insight into non-prion amyloid diseases, where interactions between amyloid aggregates (or pre-amyloid oligomers) and heterologous proteins may play a role in disease onset or progression.

Special Recognition:

EDR is supported by the NSF (MCB-1023771) and the American Heart Association (0735321N).

BIBLIOGRAPHY

1. Osherovich, L.Z. and J.S. Weissman, *Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion.* Cell, 2001. **106**(2): p. 183-94.
2. Derkatch, I.L., et al., *Prions affect the appearance of other prions: the story of [PIN(+)].* Cell, 2001. **106**(2): p. 171-82.
3. Lopez de la Paz, M. and L. Serrano, *Sequence determinants of amyloid fibril formation.* Proc Natl Acad Sci U S A, 2004. **101**(1): p. 87-92. Epub 2003 Dec 22.
4. Maurer-Stroh, S., et al., *Exploring the sequence determinants of amyloid structure using position-specific scoring matrices.* Nature Methods, 2010. **7**(3): p. 237-42.
5. Thompson, M.J., et al., *The 3D profile method for identifying fibril-forming segments of proteins.* Proc Natl Acad Sci U S A, 2006. **103**(11): p. 4074-8.
6. Stamp, J.T., et al., *Further studies on scrapie.* J Comp Pathol, 1959. **69**: p. 268-80.
7. Hadlow, W.J., *Myopathies of livestock.* Lab Invest, 1959. **8**: p. 1478-98.
8. Gajdusek, D.C. and V. Zigas, *Degenerative disease of the central nervous system in New Guinea; the endemic occurrence of kuru in the native population.* N Engl J Med, 1957. **257**(20): p. 974-8.
9. Prusiner, S.B., *Molecular biology and genetics of prion diseases.* Cold Spring Harb Symp Quant Biol, 1996. **61**: p. 473-93.
10. Aiken, J.M. and R.F. Marsh, *The search for scrapie agent nucleic acid.* Microbiol Rev, 1990. **54**(3): p. 242-6.
11. Pattison, I.H., *Resistance of the Scrapie Agent to Formalin.* J Comp Pathol, 1965. **75**: p. 159-64.
12. Alper, T., D.A. Haig, and M.C. Clarke, *The exceptionally small size of the scrapie agent.* Biochem Biophys Res Commun, 1966. **22**(3): p. 278-84.
13. Bellinger-Kawahara, C., et al., *Purified scrapie prions resist inactivation by procedures that hydrolyze, modify, or shear nucleic acids.* Virology, 1987. **160**(1): p. 271-4.
14. Griffith, J.S., *Self-replication and scrapie.* Nature, 1967. **215**(5105): p. 1043-4.
15. Bolton, D.C., M.P. McKinley, and S.B. Prusiner, *Identification of a protein that purifies with the scrapie prion.* Science, 1982. **218**(4579): p. 1309-11.
16. Prusiner, S.B., et al., *Further purification and characterization of scrapie prions.* Biochemistry, 1982. **21**(26): p. 6942-50.
17. Chesebro, B., et al., *Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain.* Nature, 1985. **315**(6017): p. 331-3.
18. Oesch, B., et al., *A cellular gene encodes scrapie PrP 27-30 protein.* Cell, 1985. **40**(4): p. 735-46.
19. Bueler, H., et al., *Mice devoid of PrP are resistant to scrapie.* Cell, 1993. **73**(7): p. 1339-47.
20. McKinley, M.P. and S.B. Prusiner, *Biology and structure of scrapie prions.* Int Rev Neurobiol, 1986. **28**: p. 1-57.
21. Kocisko, D.A., et al., *Cell-free formation of protease-resistant prion protein.* Nature, 1994. **370**(6489): p. 471-4.
22. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. **411**(6839): p. 810-3.

23. Castilla, J., et al., *In vitro generation of infectious scrapie prions.* Cell, 2005. **121**(2): p. 195-206.
24. Deleault, N.R., et al., *Formation of native prions from minimal components in vitro.* Proc Natl Acad Sci U S A, 2007. **104**(23): p. 9741-6.
25. Wang, F., et al., *Generating a prion with bacterially expressed recombinant prion protein.* Science, 2010. **327**(5969): p. 1132-5.
26. Manuelidis, L., *A 25 nm virion is the likely cause of transmissible spongiform encephalopathies.* J Cell Biochem, 2007. **100**(4): p. 897-915.
27. Manuelidis, L., *Nuclease resistant circular DNAs copurify with infectivity in scrapie and CJD.* J Neurovirol, 2011. **17**(2): p. 131-45.
28. Wuthrich, K. and R. Riek, *Three-dimensional structures of prion proteins.* Adv Protein Chem, 2001. **57**: p. 55-82.
29. Riek, R., et al., *NMR structure of the mouse prion protein domain PrP(121-231).* Nature, 1996. **382**(6587): p. 180-2.
30. Toyama, B.H., et al., *The structural basis of yeast prion strain variants.* Nature, 2007. **449**(7159): p. 233-7.
31. Collinge, J. and A.R. Clarke, *A general model of prion strains and their pathogenicity.* Science, 2007. **318**(5852): p. 930-6.
32. McKinley, M.P., D.C. Bolton, and S.B. Prusiner, *A protease-resistant protein is a structural component of the scrapie prion.* Cell, 1983. **35**(1): p. 57-62.
33. Cronier, S., et al., *Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin.* Biochem J, 2008. **416**(2): p. 297-305.
34. Lawson, V.A., et al., *N-terminal truncation of prion protein affects both formation and conformation of abnormal protease-resistant prion protein generated in vitro.* J Biol Chem, 2001. **276**(38): p. 35265-71.
35. Shewmaker, F., R.B. Wickner, and R. Tycko, *Amyloid of the prion domain of Sup35p has an in-register parallel beta-sheet structure.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19754-9.
36. Peretz, D., et al., *A conformational transition at the N terminus of the prion protein features in formation of the scrapie isoform.* J Mol Biol, 1997. **273**(3): p. 614-22.
37. Pan, K.M., et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-6.
38. Caughey, B.W., et al., *Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy.* Biochemistry, 1991. **30**(31): p. 7672-80.
39. Eanes, E.D. and G.G. Glenner, *X-ray diffraction studies on amyloid filaments.* J Histochem Cytochem, 1968. **16**(11): p. 673-7.
40. Wille, H., et al., *Natural and synthetic prion structure from X-ray fiber diffraction.* Proc Natl Acad Sci U S A, 2009. **106**(40): p. 16990-5.
41. Govaerts, C., et al., *Evidence for assembly of prions with left-handed beta-helices into trimers.* Proc Natl Acad Sci U S A, 2004. **101**(22): p. 8342-7.
42. Cobb, N.J., et al., *Molecular architecture of human prion protein amyloid: a parallel, in-register beta-structure.* Proc Natl Acad Sci U S A, 2007. **104**(48): p. 18946-51.
43. Shewmaker, F., et al., *Amyloids of shuffled prion domains that form prions have a parallel in-register beta-sheet structure.* Biochemistry, 2008. **47**(13): p. 4000-7.
44. Shewmaker, F., et al., *The functional curli amyloid is not based on in-register parallel beta-sheet structure.* J Biol Chem, 2009. **284**(37): p. 25065-76.

45. Baxa, U., et al., *Characterization of beta-sheet structure in Ure2p1-89 yeast prion fibrils by solid-state nuclear magnetic resonance.* Biochemistry, 2007. **46**(45): p. 13149-62.
46. Kryndushkin, D.S., R.B. Wickner, and R. Tycko, *The core of Ure2p prion fibrils is formed by the N-terminal segment in a parallel cross-beta structure: evidence from solid-state NMR.* J Mol Biol, 2011. **409**(2): p. 263-77.
47. Cox, B.S., *A recessive lethal super-suppressor mutation in yeast and other psi phenomena.* Heredity (Edinb), 1971. **26**(2): p. 211-32.
48. Lacroute, F., *Non-Mendelian mutation allowing ureidosuccinic acid uptake in yeast.* J Bacteriol, 1971. **106**(2): p. 519-22.
49. Drillien, R. and F. Lacroute, *Ureidosuccinic acid uptake in yeast and some aspects of its regulation.* J Bacteriol, 1972. **109**(1): p. 203-8.
50. Wickner, R.B., *[URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae.* Science, 1994. **264**(5158): p. 566-9.
51. King, C.Y., et al., *Prion-inducing domain 2-114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments.* Proc Natl Acad Sci U S A, 1997. **94**(13): p. 6618-22.
52. Taylor, K.L., et al., *Prion domain initiation of amyloid formation in vitro from native Ure2p.* Science, 1999. **283**(5406): p. 1339-43.
53. Sparrer, H.E., et al., *Evidence for the prion hypothesis: induction of the yeast [PSI+] factor by in vitro- converted Sup35 protein.* Science, 2000. **289**(5479): p. 595-9.
54. Masison, D.C. and R.B. Wickner, *Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells.* Science, 1995. **270**(5233): p. 93-5.
55. Ter-Avanesyan, M.D., et al., *The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast Saccharomyces cerevisiae.* Genetics, 1994. **137**(3): p. 671-6.
56. Li, L. and S. Lindquist, *Creating a protein-based element of inheritance.* Science, 2000. **287**(5453): p. 661-4.
57. Baxa, U., et al., *Mechanism of inactivation on prion conversion of the Saccharomyces cerevisiae Ure2 protein.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5253-60.
58. Shewmaker, F., et al., *Two prion variants of Sup35p have in-register parallel beta-sheet structures, independent of hydration.* Biochemistry, 2009. **48**(23): p. 5074-82.
59. Nelson, R., et al., *Structure of the cross-beta spine of amyloid-like fibrils.* Nature, 2005. **435**(7043): p. 773-8.
60. Ross, E.D., et al., *Primary sequence independence for prion formation.* Proc Natl Acad Sci U S A, 2005. **102**(36): p. 12825-30.
61. Ross, E.D., U. Baxa, and R.B. Wickner, *Scrambled prion domains form prions and amyloid.* Mol Cell Biol, 2004. **24**(16): p. 7206-13.
62. Krishnan, R. and S.L. Lindquist, *Structural insights into a yeast prion illuminate nucleation and strain diversity.* Nature, 2005. **435**(7043): p. 765-72.
63. Fraser, H. and A.G. Dickinson, *Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation.* J Comp Pathol, 1973. **83**(1): p. 29-40.
64. Derkatch, I.L., et al., *Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae.* Genetics, 1996. **144**(4): p. 1375-86.
65. Uptain, S.M., et al., *Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion.* EMBO J, 2001. **20**(22): p. 6236-45.

66. Kryndushkin, D.S., et al., *Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104.* J Biol Chem, 2003. **278**(49): p. 49636-43.
67. Tanaka, M., et al., *Conformational variations in an infectious protein determine prion strain differences.* Nature, 2004. **428**(6980): p. 323-8.
68. Bradley, M.E., et al., *Interactions among prions and prion "strains" in yeast.* Proc Natl Acad Sci U S A, 2002. **99 Suppl 4**: p. 16392-9.
69. Tanaka, M., et al., *The physical basis of how prion conformations determine strain phenotypes.* Nature, 2006. **442**(7102): p. 585-9.
70. Bagriantsev, S. and S.W. Liebman, *Specificity of prion assembly in vivo. [PSI+] and [PIN+] form separate structures in yeast.* J Biol Chem, 2004. **279**(49): p. 51042-8.
71. Wickner, R.B., et al., *Prion amyloid structure explains templating: how proteins can be genes.* FEMS Yeast Res, 2010. **10**(8): p. 980-91.
72. Chernoff, Y.O., et al., *Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+].* Science, 1995. **268**(5212): p. 880-4.
73. Ferreira, P.C., et al., *The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation.* Mol Microbiol, 2001. **40**(6): p. 1357-69.
74. Satpute-Krishnan, P., S.X. Langseth, and T.R. Serio, *Hsp104-dependent remodeling of prion complexes mediates protein-only inheritance.* PLoS Biol, 2007. **5**(2): p. e24.
75. Hung, G.C. and D.C. Masison, *N-terminal domain of yeast Hsp104 chaperone is dispensable for thermotolerance and prion propagation but necessary for curing prions by Hsp104 overexpression.* Genetics, 2006. **173**(2): p. 611-20.
76. Tessarz, P., A. Mogk, and B. Bukau, *Substrate threading through the central pore of the Hsp104 chaperone as a common mechanism for protein disaggregation and prion propagation.* Mol Microbiol, 2008. **68**(1): p. 87-97.
77. Bagriantsev, S.N., et al., *Variant-specific [PSI+] infection is transmitted by Sup35 polymers within [PSI+] aggregates with heterogeneous protein composition.* Mol Biol Cell, 2008. **19**(6): p. 2433-43.
78. Hines, J.K., et al., *Influence of prion variant and yeast strain variation on prion-molecular chaperone requirements.* Prion, 2011. **5**(4): p. 238-44.
79. Jones, G.W., Y. Song, and D.C. Masison, *Deletion of the Hsp70 chaperone gene SSB causes hypersensitivity to guanidine toxicity and curing of the [PSI+] prion by increasing guanidine uptake in yeast.* Mol Genet Genomics, 2003. **269**(3): p. 304-11.
80. Jung, G., et al., *A role for cytosolic hsp70 in yeast [PSI(+)] prion propagation and [PSI(+)] as a cellular stress.* Genetics, 2000. **156**(2): p. 559-70.
81. Chacinska, A., et al., *Ssb1 chaperone is a [PSI+] prion-curing factor.* Curr Genet, 2001. **39**(2): p. 62-7.
82. Chernoff, Y.O., et al., *Evidence for a protein mutator in yeast: role of the Hsp70-related chaperone ssb in formation, stability, and toxicity of the [PSI] prion.* Mol Cell Biol, 1999. **19**(12): p. 8103-12.
83. Kushnirov, V.V., et al., *Chaperones that cure yeast artificial [PSI+] and their prion-specific effects.* Curr Biol, 2000. **10**(22): p. 1443-6.
84. Aron, R., et al., *J-protein co-chaperone Sis1 required for generation of [RNQ+] seeds necessary for prion propagation.* EMBO J, 2007. **26**(16): p. 3794-803.
85. Sondheimer, N., et al., *The role of Sis1 in the maintenance of the [RNQ+] prion.* EMBO J, 2001. **20**(10): p. 2435-42.

86. Gonzalez Nelson, A.C. and E.D. Ross, *Interactions between non-identical prion proteins.* Semin Cell Dev Biol, 2011. **22**(5): p. 437-43.
87. Derkatch, I.L., et al., *Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae.* Genetics, 1997. **147**(2): p. 507-19.
88. Sondheimer, N. and S. Lindquist, *Rnq1: an epigenetic modifier of protein function in yeast.* Mol Cell, 2000. **5**(1): p. 163-72.
89. Saupe, S.J., *The [Het-s] prion of Podospora anserina and its role in heterokaryon incompatibility.* Semin Cell Dev Biol, 2011. **22**(5): p. 460-8.
90. Wasmer, C., et al., *Amyloid fibrils of the HET-s(218-289) prion form a beta solenoid with a triangular hydrophobic core.* Science, 2008. **319**(5869): p. 1523-6.
91. Lange, A., et al., *A combined solid-state NMR and MD characterization of the stability and dynamics of the HET-s(218-289) prion in its amyloid conformation.* Chembiochem, 2009. **10**(10): p. 1657-65.
92. Santoso, A., et al., *Molecular basis of a yeast prion species barrier.* Cell, 2000. **100**(2): p. 277-88.
93. Derkatch, I.L., et al., *Dependence and independence of [PSI(+)] and [PIN(+)]: a two-prion system in yeast?* EMBO J, 2000. **19**(9): p. 1942-52.
94. Li, Z., et al., *Rational extension of the ribosome biogenesis pathway using network-guided genetics.* PLoS Biol, 2009. **7**(10): p. e1000213.
95. Michelitsch, M.D. and J.S. Weissman, *A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions.* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 11910-5.
96. Smith, C.L., et al., *Structural analysis of the yeast SWI/SNF chromatin remodeling complex.* Nat Struct Biol, 2003. **10**(2): p. 141-5.
97. Du, Z., et al., *Newly identified prion linked to the chromatin-remodeling factor Swi1 in Saccharomyces cerevisiae.* Nat Genet, 2008. **40**(4): p. 460-5.
98. DeRisi, J.L., V.R. Iyer, and P.O. Brown, *Exploring the metabolic and genetic control of gene expression on a genomic scale.* Science, 1997. **278**(5338): p. 680-6.
99. Green, S.R. and A.D. Johnson, *Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in Saccharomyces cerevisiae.* Mol Biol Cell, 2004. **15**(9): p. 4191-202.
100. Smith, R.L. and A.D. Johnson, *Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes.* Trends Biochem Sci, 2000. **25**(7): p. 325-30.
101. Rothstein, R.J. and F. Sherman, *Genes affecting the expression of cytochrome c in yeast: genetic mapping and genetic interactions.* Genetics, 1980. **94**(4): p. 871-89.
102. Neigeborn, L. and M. Carlson, *Genes affecting the regulation of SUC2 gene expression by glucose repression in Saccharomyces cerevisiae.* Genetics, 1984. **108**(4): p. 845-58.
103. Patel, B.K., J. Gavin-Smyth, and S.W. Liebman, *The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion.* Nat Cell Biol, 2009. **11**(3): p. 344-9.
104. Grishin, A.V., et al., *Mot3, a Zn finger transcription factor that modulates gene expression and attenuates mating pheromone signaling in Saccharomyces cerevisiae.* Genetics, 1998. **149**(2): p. 879-92.
105. Alberti, S., et al., *A systematic survey identifies prions and illuminates sequence features of prionogenic proteins.* Cell, 2009. **137**(1): p. 146-58.

106. Xu, Z. and D. Norris, *The SFP1 gene product of Saccharomyces cerevisiae regulates G2/M transitions during the mitotic cell cycle and DNA-damage response.* Genetics, 1998. **150**(4): p. 1419-28.
107. Jorgensen, P., et al., *A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size.* Genes Dev, 2004. **18**(20): p. 2491-505.
108. Marion, R.M., et al., *Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression.* Proc Natl Acad Sci U S A, 2004. **101**(40): p. 14315-22.
109. Volkov, K.V., et al., *Novel non-Mendelian determinant involved in the control of translation accuracy in Saccharomyces cerevisiae.* Genetics, 2002. **160**(1): p. 25-36.
110. Rogoza, T., et al., *Non-Mendelian determinant [ISP+] in yeast is a nuclear-residing prion form of the global transcriptional regulator Sfp1.* Proc Natl Acad Sci U S A, 2010. **107**(23): p. 10573-7.
111. Saifitdinova, A.F., et al., *[NSI (+)]: a novel non-Mendelian nonsense suppressor determinant in Saccharomyces cerevisiae.* Curr Genet, 2010. **56**(5): p. 467-78.
112. Nizhnikov, A.A., et al., *[NSI+] determinant has a pleiotropic phenotypic manifestation that is modulated by SUP35, SUP45, and VTS1 genes.* Curr Genet, 2012. **58**(1): p. 35-47.
113. Dihanich, M.E., et al., *Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of Saccharomyces cerevisiae.* Mol Cell Biol, 1987. **7**(1): p. 177-84.
114. Suzuki, G., N. Shimazu, and M. Tanaka, *A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress.* Science, 2012. **336**(6079): p. 355-9.
115. Harrison, P.M. and M. Gerstein, *A method to assess compositional bias in biological sequences and its application to prion-like glutamine/asparagine-rich domains in eukaryotic proteomes.* Genome Biol, 2003. **4**(6): p. R40.
116. Bryan, A.W., Jr., et al., *BETASCAN: probable beta-amyloids identified by pairwise probabilistic analysis.* PLoS Comput Biol, 2009. **5**(3): p. e1000333.
117. Fernandez-Escamilla, A.M., et al., *Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins.* Nat Biotechnol, 2004. **22**(10): p. 1302-6.
118. Tartaglia, G.G. and M. Vendruscolo, *The Zyggregator method for predicting protein aggregation propensities.* Chem Soc Rev, 2008. **37**(7): p. 1395-401.
119. Zibaee, S., et al., *A simple algorithm locates beta-strands in the amyloid fibril core of alpha-synuclein, Abeta, and tau using the amino acid sequence alone.* Protein Sci, 2007. **16**(5): p. 906-18.
120. Trovato, A., F. Seno, and S.C. Tosatto, *The PASTA server for protein aggregation prediction.* Protein Eng Des Sel, 2007. **20**(10): p. 521-3.
121. Toombs, J.A., B.R. McCarty, and E.D. Ross, *Compositional determinants of prion formation in yeast.* Mol Cell Biol, 2010. **30**(1): p. 319-32.
122. Ross, E.D. and J.A. Toombs, *The effects of amino acid composition on yeast prion formation and prion domain interactions.* Prion, 2010. **4**(2): p. 60-5.
123. Uversky, V.N., J.R. Gillespie, and A.L. Fink, *Why are "natively unfolded" proteins unstructured under physiologic conditions?* Proteins, 2000. **41**(3): p. 415-27.
124. Alexandrov, A.I., et al., *The effects of amino acid composition of glutamine-rich domains on amyloid formation and fragmentation.* PLoS ONE, 2012. **7**(10): p. e46458.

125. Gonzalez Nelson, A.C., et al., *Increasing prion propensity by hydrophobic insertion.* PLoS One, 2014. **9**(2): p. e89286.
126. Wickner, R.B., et al., *The yeast prions [PSI+] and [URE3] are molecular degenerative diseases.* Prion, 2011. **5**(4): p. 258-62.
127. Halfmann, R. and S. Lindquist, *Epigenetics in the extreme: prions and the inheritance of environmentally acquired traits.* Science, 2010. **330**(6004): p. 629-32.
128. Halfmann, R., S. Alberti, and S. Lindquist, *Prions, protein homeostasis, and phenotypic diversity.* Trends Cell Biol, 2010. **20**(3): p. 125-33.
129. Resende, C.G., et al., *Prion protein gene polymorphisms in Saccharomyces cerevisiae.* Mol Microbiol, 2003. **49**(4): p. 1005-17.
130. Chernoff, Y.O., et al., *Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein.* Mol Microbiol, 2000. **35**(4): p. 865-76.
131. Nakayashiki, T., et al., *Yeast prions [URE3] and [PSI+] are diseases.* Proc Natl Acad Sci U S A, 2005. **102**(30): p. 10575-80.
132. Halfmann, R., et al., *Prions are a common mechanism for phenotypic inheritance in wild yeasts.* Nature, 2012. **482**(7385): p. 363-8.
133. Edskes, H.K., et al., *Prion-forming ability of Ure2 of yeasts is not evolutionarily conserved.* Genetics, 2011. **188**(1): p. 81-90.
134. Kushnirov, V.V., et al., *Prion properties of the Sup35 protein of yeast Pichia methanolica.* EMBO J, 2000. **19**(3): p. 324-31.
135. Baudin-Baillieu, A., et al., *Conservation of the prion properties of Ure2p through evolution.* Mol Biol Cell, 2003. **14**(8): p. 3449-58.
136. Harrison, L.B., et al., *Evolution of budding yeast prion-determinant sequences across diverse fungi.* J Mol Biol, 2007. **368**(1): p. 273-82.
137. Shewmaker, F., et al., *Ure2p function is enhanced by its prion domain in Saccharomyces cerevisiae.* Genetics, 2007. **176**(3): p. 1557-65.
138. Hosoda, N., et al., *Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation.* J Biol Chem, 2003. **278**(40): p. 38287-91.
139. Bailleul, P.A., et al., *Genetic study of interactions between the cytoskeletal assembly protein sla1 and prion-forming domain of the release factor Sup35 (eRF3) in Saccharomyces cerevisiae.* Genetics, 1999. **153**(1): p. 81-94.
140. Tartaglia, G.G., et al., *Life on the edge: a link between gene expression levels and aggregation rates of human proteins.* Trends Biochem Sci, 2007. **32**(5): p. 204-6.
141. Paul, K.R. and E.D. Ross, *Controlling the prion propensity of glutamine/asparagine-rich proteins.* Prion, 2015. **9**(5): p. 347-54.
142. True, H.L. and S.L. Lindquist, *A yeast prion provides a mechanism for genetic variation and phenotypic diversity.* Nature, 2000. **407**(6803): p. 477-83.
143. McGlinchey, R.P., D. Kryndushkin, and R.B. Wickner, *Suicidal [PSI+] is a lethal yeast prion.* Proc Natl Acad Sci U S A, 2011. **108**(13): p. 5337-41.
144. Tyedmers, J., M.L. Madariaga, and S. Lindquist, *Prion switching in response to environmental stress.* PLoS Biol, 2008. **6**(11): p. e294.
145. Hou, F., et al., *MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response.* Cell, 2011. **146**(3): p. 448-61.
146. Wang, X., et al., *In vitro polymerization of a functional Escherichia coli amyloid protein.* J Biol Chem, 2007. **282**(6): p. 3713-9.

147. White, A.P., et al., *Structure and characterization of AgfB from Salmonella enteritidis thin aggregative fimbriae.* J Mol Biol, 2001. **311**(4): p. 735-49.
148. Chapman, M.R., et al., *Role of Escherichia coli curli operons in directing amyloid fiber formation.* Science, 2002. **295**(5556): p. 851-5.
149. Shu, Q., et al., *The E. coli CsgB nucleator of curli assembles to beta-sheet oligomers that alter the CsgA fibrillization mechanism.* Proc Natl Acad Sci U S A, 2012. **109**(17): p. 6502-7.
150. Anderson, P. and N. Kedersha, *Visibly stressed: the role of eIF2, TIA-1, and stress granules in protein translation.* Cell Stress Chaperones, 2002. **7**(2): p. 213-21.
151. Gilks, N., et al., *Stress granule assembly is mediated by prion-like aggregation of TIA-1.* Mol Biol Cell, 2004. **15**(12): p. 5383-98.
152. Wadsworth, J.D. and J. Collinge, *Update on human prion disease.* Biochim Biophys Acta, 2007. **1772**(6): p. 598-609.
153. Coustou, V., et al., *The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog.* Proc. Natl. Acad. Sci. USA, 1997. **94**(18): p. 9773-8.
154. Maclea, K.S. and E.D. Ross, *Strategies for identifying new prions in yeast.* Prion, 2011. **5**(4): p. 263-268.
155. Li, Y.R., et al., *Stress granules as crucibles of ALS pathogenesis.* J Cell Biol, 2013. **201**(3): p. 361-72.
156. Da Cruz, S. and D.W. Cleveland, *Understanding the role of TDP-43 and FUS/TLS in ALS and beyond.* Curr Opin Neurobiol, 2011. **21**(6): p. 904-19.
157. Geser, F., et al., *Amyotrophic lateral sclerosis, frontotemporal dementia and beyond: the TDP-43 diseases.* J Neurol, 2009. **256**(8): p. 1205-14.
158. Weihl, C.C., et al., *TDP-43 accumulation in inclusion body myopathy muscle suggests a common pathogenic mechanism with frontotemporal dementia.* J Neurol Neurosurg Psychiatry, 2008. **79**(10): p. 1186-9.
159. Couthouis, J., et al., *Evaluating the role of the FUS/TLS-related gene EWSR1 in amyotrophic lateral sclerosis.* Hum Mol Genet, 2012: p. Epub ahead of print.
160. Couthouis, J., et al., *A yeast functional screen predicts new candidate ALS disease genes.* Proc Natl Acad Sci U S A, 2011. **108**: p. 20881-20890.
161. Neumann, M., et al., *FET proteins TAF15 and EWS are selective markers that distinguish FTLD with FUS pathology from amyotrophic lateral sclerosis with FUS mutations.* Brain, 2011. **134**(Pt 9): p. 2595-609.
162. Kim, H.J., et al., *Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS.* Nature, 2013. **495**(7442): p. 467-73.
163. Klar, J., et al., *Welander distal myopathy caused by an ancient founder mutation in TIA1 associated with perturbed splicing.* Hum Mutat, 2013. **34**(4): p. 572-7.
164. Bradley, M.E. and S.W. Liebman, *The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI+] prions.* Mol. Microbiol., 2004. **51**(6): p. 1649-1659.
165. Ter-Avanesyan, M.D., et al., *Deletion analysis of the SUP35 gene of the yeast Saccharomyces cerevisiae reveals two non-overlapping functional regions in the encoded protein.* Mol. Microbiol., 1993. **7**(5): p. 683-92.

166. Harrison, P.M. and M. Gerstein, *A method to assess compositional bias in biological sequences and its application to prion-like glutamine/asparagine-rich domains in eukaryotic proteomes.* Genome Biol, 2003. **4**(6): p. R40.
167. Liu, J.J., N. Sondheimer, and S.L. Lindquist, *Changes in the middle region of Sup35 profoundly alter the nature of epigenetic inheritance for the yeast prion [PSI+].* Proc. Natl. Acad. Sci. USA, 2002. **99 Suppl 4**: p. 16446-53.
168. Ross, E.D., U. Baxa, and R.B. Wickner, *Scrambled Prion Domains Form Prions and Amyloid.* Mol. Cell. Biol., 2004. **24**(16): p. 7206-7213.
169. Michelitsch, M.D. and J.S. Weissman, *A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions.* Proc. Natl. Acad. Sci. USA, 2000. **97**(22): p. 11910-5.
170. Sondheimer, N. and S. Lindquist, *Rnq1: an epigenetic modifier of protein function in yeast.* Mol. Cell, 2000. **5**(1): p. 163-72.
171. Halfmann, R., et al., *Prion formation by a yeast GLFG nucleoporin.* Prion, 2012. **6**(4).
172. Toombs, J.A., B.R. McCarty, and E.D. Ross, *Compositional determinants of prion formation in yeast.* Mol. Cell. Biol., 2010. **30**(1): p. 319-332.
173. Ross, E.D., et al., *A bioinformatics method for identifying Q/N-rich prion-like domains in proteins.* Methods Mol Biol, 2013. **1017**: p. 219-28.
174. Toombs, J.A., et al., *De novo design of synthetic prion domains.* Proc Natl Acad Sci U S A, 2012. **109**(17): p. 6519-6524.
175. Chiti, F., et al., *Rationalization of the effects of mutations on peptide and protein aggregation rates.* Nature, 2003. **424**(6950): p. 805-8.
176. Tartaglia, G.G., et al., *Prediction of aggregation-prone regions in structured proteins.* J Mol Biol, 2008. **380**(2): p. 425-36.
177. Goldschmidt, L., et al., *Identifying the amylome, proteins capable of forming amyloid-like fibrils.* Proc. Natl. Acad. Sci. USA 2010. **107**(8): p. 3487-3492.
178. Fernandez-Escamilla, A.M., et al., *Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins.* Nat. Biotechnol., 2004. **22**(10): p. 1302-6.
179. Salnikova, A.B., et al., *Nonsense suppression in yeast cells overproducing Sup35 (eRF3) is caused by its non-heritable amyloids.* J Biol Chem, 2005. **280**(10): p. 8808-12.
180. Toombs, J.A., et al., *[PSI+] maintenance is dependent on the composition, not primary sequence, of the oligopeptide repeat domain. PLoS One*, 2011. **6**(7): p. e21953.
181. Sherman, F., *Getting started with yeast.* Methods Enzymol., 1991. **194**: p. 3-21.
182. Song, Y., et al., *Role for Hsp70 chaperone in Saccharomyces cerevisiae prion seed replication.* Eukaryot. Cell, 2005. **4**(2): p. 289-97.
183. Ross, E.D., et al., *Primary sequence independence for prion formation.* Proc. Natl. Acad. Sci. USA, 2005. **102**(36): p. 12825-12830.
184. Gietz, R.D. and A. Sugino, *New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites.* Gene, 1988. **74**(2): p. 527-34.
185. Cormack, B.P., et al., *Yeast-enhanced green fluorescent protein (yEGFP)a reporter of gene expression in Candida albicans.* Microbiology, 1997. **143**(Pt 2): p. 303-11.
186. Ross, C.D., et al., *A promiscuous prion: Efficient induction of [URE3] prion formation by heterologous prion domains.* Genetics, 2009. **183**(3): p. 929-40.

187. Bagriantsev, S.N., V.V. Kushnirov, and S.W. Liebman, *Analysis of amyloid aggregates using agarose gel electrophoresis.* Methods Enzymol, 2006. **412**: p. 33-48.
188. Collins, S.R., et al., *Mechanism of Prion Propagation: Amyloid Growth Occurs by Monomer Addition.* PLoS Biology, 2004. **2**(10): p. e321.
189. Alexandrov, I.M., et al., *Appearance and propagation of polyglutamine-based amyloids in yeast: tyrosine residues enable polymer fragmentation.* J Biol Chem, 2008. **283**(22): p. 15185-92.
190. Osherovich, L.Z., et al., *Dissection and design of yeast prions.* PLoS Biol., 2004. **2**(4): p. E86.
191. DePace, A.H., et al., *A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion.* Cell, 1998. **93**(7): p. 1241-52.
192. Parham, S.N., C.G. Resende, and M.F. Tuite, *Oligopeptide repeats in the yeast protein Sup35p stabilize intermolecular prion interactions.* EMBO J., 2001. **20**(9): p. 2111-9.
193. Shkundina, I.S., et al., *The role of the N-terminal oligopeptide repeats of the yeast sup35 prion protein in propagation and transmission of prion variants.* Genetics, 2006. **172**(2): p. 827-35.
194. Cox, B.S., *PSI, a cytoplasmic suppressor of super-suppressor in yeast.* Heredity, 1965. **26**: p. 211-232.
195. Lancaster, A.K., et al., *The spontaneous appearance rate of the yeast prion [PSI+] and its implications for the evolution of the evolvability properties of the [PSI+] system.* Genetics, 2010. **184**(2): p. 393-400.
196. Derkatch, I.L., et al., *Genetic and Environmental Factors Affecting the de novo Appearance of the [PSI(+)] Prion in Saccharomyces cerevisiae.* Genetics, 1997. **147**(2): p. 507-519.
197. Ferreira, P.C., et al., *The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation.* Mol. Microbiol., 2001. **40**(6): p. 1357-69.
198. Jung, G. and D.C. Masison, *Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions.* Curr. Microbiol., 2001. **43**(1): p. 7-10.
199. Ness, F., et al., *Guanidine hydrochloride inhibits the generation of prion "seeds" but not prion protein aggregation in yeast.* Mol. Cell. Biol., 2002. **22**(15): p. 5593-605.
200. Paushkin, S.V., et al., *Propagation of the yeast prion-like [psi+] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor.* EMBO J., 1996. **15**(12): p. 3127-34.
201. Wegrzyn, R.D., et al., *Mechanism of prion loss after Hsp104 inactivation in yeast.* Mol. Cell. Biol., 2001. **21**(14): p. 4656-69.
202. LeVine, H., 3rd, *Quantification of beta-sheet amyloid fibril structures with thioflavin T.* Methods Enzymol, 1999. **309**: p. 274-84.
203. Chen, B., et al., *Genetic and epigenetic control of the efficiency and fidelity of cross-species prion transmission.* Mol Microbiol, 2010. **76**(6): p. 1483-99.
204. Santoso, A., et al., *Molecular basis of a yeast prion species barrier.* Cell, 2000. **100**(2): p. 277-88.
205. Tessier, P.M. and S. Lindquist, *Prion recognition elements govern nucleation, strain specificity and species barriers.* Nature, 2007. **447**(7144): p. 556-61. Epub 2007 May 9.

206. Pastor, M.T., A. Esteras-Chopo, and L. Serrano, *Hacking the code of amyloid formation: the amyloid stretch hypothesis.* Prion, 2007. **1**(1): p. 9-14. Epub 2007 Jan 5.
207. Teng, P.K. and D. Eisenberg, *Short protein segments can drive a non-fibrillizing protein into the amyloid state.* Protein Eng Des Sel, 2009. **22**(8): p. 531-6.
208. Ohhashi, Y., et al., *Differences in prion strain conformations result from non-native interactions in a nucleus.* Nature, 2010. **6**(3): p. 225-230.
209. Street, A.G. and S.L. Mayo, *Intrinsic beta-sheet propensities result from van der Waals interactions between side chains and the local backbone.* Proc Natl Acad Sci U S A, 1999. **96**(16): p. 9074-6.
210. Espinosa Angarica, V., S. Ventura, and J. Sancho, *Discovering putative prion sequences in complete proteomes using probabilistic representations of Q/N-rich domains.* BMC Genomics, 2013. **14**: p. 316.
211. Crow, E.T., Z. Du, and L. Li, *A small, glutamine-free domain propagates the [SWI(+)] prion in budding yeast.* Mol Cell Biol, 2011. **31**(16): p. 3436-44.
212. Vitrenko, Y.A., et al., *Propagation of the [PIN+] prion by fragments of Rnq1 fused to GFP.* Curr Genet, 2007. **51**(5): p. 309-19.
213. Zhou, P., I.L. Derkatch, and S.W. Liebman, *The relationship between visible intracellular aggregates that appear after overexpression of Sup35 and the yeast prion-like elements [PSI(+)] and [PIN(+)].* Mol Microbiol, 2001. **39**(1): p. 37-46.
214. Fernandez-Bellot, E., E. Guillemet, and C. Cullin, *The yeast prion [URE3] can be greatly induced by a functional mutated URE2 allele.* EMBO J, 2000. **19**(13): p. 3215-22.
215. Kawai-Noma, S., et al., *Dynamics of yeast prion aggregates in single living cells.* Genes Cells, 2006. **11**(9): p. 1085-96.
216. Masison, D.C., M.L. Maddelein, and R.B. Wickner, *The prion model for [URE3] of yeast: spontaneous generation and requirements for propagation.* Proc Natl Acad Sci U S A, 1997. **94**(23): p. 12503-8.
217. Yu, Y., et al., *Flexibility of the Ure2 prion domain is important for amyloid fibril formation.* Biochem J, 2011. **434**(1): p. 143-51.
218. Moerner, W.E., et al., *Optical methods for exploring dynamics of single copies of green fluorescent protein.* Cytometry, 1999. **36**(3): p. 232-8.
219. Edskes, H.K., V.T. Gray, and R.B. Wickner, *The [URE3] prion is an aggregated form of Ure2p that can be cured by overexpression of Ure2p fragments.* Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1498-503.
220. Sherman, F., *Getting started with yeast.* Methods Enzymol, 1991. **194**: p. 3-21.
221. Ranson, N., et al., *Insights into the architecture of the Ure2p yeast protein assemblies from helical twisted fibrils.* Protein Sci, 2006. **15**(11): p. 2481-7.
222. Kodama, Y. and C.D. Hu, *Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives.* Biotechniques, 2012. **53**(5): p. 285-98.
223. Futcher, A.B. and B.S. Cox, *Copy number and the stability of 2-micron circle-based artificial plasmids of Saccharomyces cerevisiae.* J Bacteriol, 1984. **157**(1): p. 283-90.
224. Kajava, A.V., et al., *A model for Ure2p prion filaments and other amyloids: the parallel superpleated beta-structure.* Proc Natl Acad Sci U S A, 2004. **101**(21): p. 7885-90.
225. Wetzel, R., *Mutations and off-pathway aggregation of proteins.* Trends Biotechnol, 1994. **12**(5): p. 193-8.
226. Harbi, D. and P.M. Harrison, *Classifying prion and prion-like phenomena.* Prion, 2014. **8**(2).

227. Harbi, D. and P.M. Harrison, *Interaction networks of prion, prionogenic and prion-like proteins in budding yeast, and their role in gene regulation.* PLoS One, 2014. **9**(6): p. e100615.
228. Song, Y., et al., *Quantitative effects of magnesium chloride stress on aggregation of Sup35p in [psi-] yeast cells.* Protein Pept Lett, 2010. **17**(12): p. 1489-94.
229. Richter, K., M. Haslbeck, and J. Buchner, *The heat shock response: life on the verge of death.* Mol Cell, 2010. **40**(2): p. 253-66.
230. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. **216**(4542): p. 136-44.
231. Aguzzi, A. and M. Polymenidou, *Mammalian prion biology: one century of evolving concepts.* Cell, 2004. **116**(2): p. 313-27.
232. Wickner, R.B., et al., *Protein inheritance (prions) based on parallel in-register beta-sheet amyloid structures.* Bioessays, 2008. **30**(10): p. 955-64.
233. Pierce, M.M., et al., *Is the prion domain of soluble Ure2p unstructured?* Biochemistry, 2005. **44**(1): p. 321-8.
234. Serio, T.R., et al., *Nucleated conformational conversion and the replication of conformational information by a prion determinant.* Science, 2000. **289**(5483): p. 1317-21.
235. Wickner, R.B., et al., *Prions of yeast and fungi. Proteins as genetic material.* J. Biol. Chem., 1999. **274**(2): p. 555-8.
236. Wickner, R.B., *A new prion controls fungal cell fusion incompatibility.* Proc Natl Acad Sci U S A, 1997. **94**(19): p. 10012-4.
237. Eaglestone, S.S., B.S. Cox, and M.F. Tuite, *Translation termination efficiency can be regulated in Saccharomyces cerevisiae by environmental stress through a prion-mediated mechanism.* EMBO J, 1999. **18**(7): p. 1974-81.
238. Tyedmers, J., M.L. Madariaga, and S. Lindquist, *Prion switching in response to environmental stress.* PLoS Biol, 2008. **6**(11): p. e294.
239. Tycko, R., et al., *The alpha-Helical C-Terminal Domain of Full-Length Recombinant PrP Converts to an In-Register Parallel beta-Sheet Structure in PrP Fibrils: Evidence from Solid State Nuclear Magnetic Resonance.* Biochemistry, 2010: p. Epub ahead of print.
240. Baxa, U., et al., *Characterization of beta-Sheet Structure in Ure2p1-89 Yeast Prion Fibrils by Solid-State Nuclear Magnetic Resonance.* Biochemistry, 2007. **46**(45): p. 13149-13162.
241. Wickner, R.B., F. Dyda, and R. Tycko, *Amyloid of Rnq1p, the basis of the [PIN+] prion, has a parallel in-register beta-sheet structure.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2403-8.
242. Hilbich, C., et al., *Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease.* J Mol Biol, 1991. **218**(1): p. 149-63.
243. Petkova, A.T., et al., *A structural model for Alzheimer's beta-amyloid fibrils based on experimental constraints from solid state NMR.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(26): p. 16742-16747.
244. Perutz, M.F., et al., *Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases.* Proc Natl Acad Sci U S A, 1994. **91**(12): p. 5355-8.
245. Rikhvanov, E.G., N.V. Romanova, and Y.O. Chernoff, *Chaperone effects on prion and nonprion aggregates.* Prion, 2007. **1**(4): p. 217-22. Epub 2007 Oct 6.

246. Romanova, N.V. and Y.O. Chernoff, *Hsp104 and prion propagation.* Protein Pept Lett, 2009. **16**(6): p. 598-605.
247. Pattison, I.H., *Experiments with scrapie with special reference to the nature of the agent and the pathology of the disease*, in *Slow, Latent and Temperate Virus Infections, NINDB Monograph 2*, D.C. Gajdusek, C.J. Gibbs, and M.P. Alpers, Editors. 1965, US Government Printing Office: Washington, DC. p. 249–257.
248. Nakayashiki, T., et al., *Yeast [PSI+] "prions" that are crosstransmissible and susceptible beyond a species barrier through a quasi-prion state.* Mol Cell, 2001. **7**(6): p. 1121-30.
249. Chen, B., G.P. Newnam, and Y.O. Chernoff, *Prion species barrier between the closely related yeast proteins is detected despite coaggregation.* Proc Natl Acad Sci U S A, 2007. **104**(8): p. 2791-6.
250. Chernoff, Y.O., et al., *Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein.* Mol. Microbiol., 2000. **35**(4): p. 865-76.
251. Scott, M., et al., *Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques.* Cell, 1989. **59**(5): p. 847-57.
252. Caughey, B., G.J. Raymond, and R.A. Bessen, *Strain-dependent differences in beta-sheet conformations of abnormal prion protein.* J Biol Chem, 1998. **273**(48): p. 32230-5.
253. Safar, J., et al., *Eight prion strains have PrP(Sc) molecules with different conformations.* Nat Med, 1998. **4**(10): p. 1157-65.
254. Hill, A.F., et al., *The same prion strain causes vCJD and BSE.* Nature, 1997. **389**(6650): p. 448-50, 526.
255. Flechsig, E. and C. Weissmann, *The role of PrP in health and disease.* Curr Mol Med, 2004. **4**(4): p. 337-53.
256. Makarava, N., et al., *Highly promiscuous nature of prion polymerization.* J Biol Chem, 2007. **282**(50): p. 36704-13.
257. King, C.Y. and R. Diaz-Avalos, *Protein-only transmission of three yeast prion strains.* Nature, 2004. **428**(6980): p. 319-23.
258. Bruce, K.L. and Y.O. Chernoff, *Sequence specificity and fidelity of prion transmission in yeast.* Seminars in Cell and Developmental Biology, 2011. **(this issue)**.
259. Liu, J.J. and S. Lindquist, *Oligopeptide-repeat expansions modulate 'protein-only' inheritance in yeast.* Nature, 1999. **400**(6744): p. 573-6.
260. Chien, P. and J.S. Weissman, *Conformational diversity in a yeast prion dictates its seeding specificity.* Nature, 2001. **410**(6825): p. 223-7.
261. Chien, P., et al., *Generation of prion transmission barriers by mutational control of amyloid conformations.* Nature, 2003. **424**(6951): p. 948-51.
262. Edskes, H.K., et al., *Prion variants and species barriers among Saccharomyces Ure2 proteins.* Genetics, 2009. **181**(3): p. 1159-67. Epub 2009 Jan 5.
263. Mukhopadhyay, S., et al., *A natively unfolded yeast prion monomer adopts an ensemble of collapsed and rapidly fluctuating structures.* Proc Natl Acad Sci U S A, 2007. **104**(8): p. 2649-54. Epub 2007 Feb 13.
264. Tuite, M.F., C.R. Mundy, and B.S. Cox, *Agents that cause a high frequency of genetic change from [psi+] to [psi-] in Saccharomyces cerevisiae.* Genetics, 1981. **98**(4): p. 691-711.
265. Derkatch, I.L., et al., *Dependence and independence of [PSI(+)] and [PIN(+)]: a two-prion system in yeast?* EMBO J., 2000. **19**(9): p. 1942-52.

266. Derkatch, I.L., et al., *Effects of Q/N-rich, polyQ, and non-polyQ amyloids on the de novo formation of the [PSI+] prion in yeast and aggregation of Sup35 in vitro.* Proc. Natl. Acad. Sci. USA, 2004. **101**(35): p. 12934-12939.
267. Choe, Y.J., et al., *Increased [PSI+] appearance by fusion of Rnq1 with the prion domain of Sup35 in Saccharomyces cerevisiae.* Eukaryot Cell, 2009. **8**(7): p. 968-976.
268. Ganusova, E.E., et al., *Modulation of prion formation, aggregation, and toxicity by the actin cytoskeleton in yeast.* Mol Cell Biol, 2006. **26**(2): p. 617-29.
269. Bagriantsev, S. and S.W. Liebman, *Specificity of Prion Assembly in Vivo: [PSI+] AND [PIN+] form separate structures in yeast.* J. Biol. Chem., 2004. **279**(49): p. 51042-51048.
270. Bradley, M.E., et al., *Interactions among prions and prion "strains" in yeast.* Proc. Natl. Acad. Sci. USA, 2002. **99 Suppl 4**: p. 16392-9.
271. Vitrenko, Y.A., et al., *Propagation of the [PIN+] prion by fragments of Rnq1 fused to GFP.* Curr Genet, 2007. **51**(5): p. 309-19. Epub 2007 Apr 6.
272. Kadnar, M.L., G. Articov, and I.L. Derkatch, *Distinct type of transmission barrier revealed by study of multiple prion determinants of Rnq1.* PLoS, 2010. **6**(1): p. e1000824.
273. Bardill, J.P. and H.L. True, *Heterologous prion interactions are altered by mutations in the prion protein Rnq1p.* J Mol Biol, 2009. **388**(3): p. 583-96. Epub 2009 Mar 24.
274. Vishveshwara, N. and S.W. Liebman, *Heterologous cross-seeding mimics cross-species prion conversion in a yeast model.* BMC Biol, 2009. **7**: p. 26.
275. Schwimmer, C. and D.C. Masison, *Antagonistic interactions between yeast [PSI(+)] and [URE3] prions and curing of [URE3] by Hsp70 protein chaperone Ssa1p but not by Ssa2p.* Mol. Cell. Biol., 2002. **22**(11): p. 3590-8.
276. Bradley, M.E. and S.W. Liebman, *Destabilizing interactions among [PSI(+)] and [PIN(+)] yeast prion variants.* Genetics, 2003. **165**(4): p. 1675-85.

ADDENDUM 2: GARRF SCRIPT

```
# Copyright (C) 2003, 2004 by BiRC -- Bioinformatics Research Center
#                          University of Aarhus, Denmark
#                          Contact: Thomas Mailund <mailund@birc.dk>
#
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Adapted from : A parser for FASTA files provided by Asa Ben-Hur

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                         University of Aarhus, Denmark
                         Contact: Thomas Mailund <mailund@birc.dk>
"""

from __future__ import generators
import os
```

```
def myopen(fileName) :

    if not ( os.path.exists(fileName) and os.path.isfile(fileName) ):
        raise ValueError, 'file does not exist at %s' % fileName

    import gzip
    fileHandle = gzip.GzipFile(fileName)

    gzippedFile = True
    try :
        line = fileHandle.readline()
        fileHandle.close()
    except :
        gzippedFile = False

    if gzippedFile :
        return gzip.GzipFile(fileName)
    else :
        return open(fileName)


class MalformedInput :
    "Exception raised when the input file does not look like a fasta file."
    pass

class FastaRecord :
    "a fasta record."

    def __init__(self, header, sequence):
```

```
        "Create a record with the given header and sequence."
        self.header = header
        self.sequence = sequence

    def __str__(self) :

        return '>' + self.header + '\n' + self.sequence + '\n'


def _fasta_itr_from_file(file) :
    "Provide an iteration through the fasta records in file."

    h = file.readline()[:-1]
    if h[0] != '>':
        raise MalformedInput()
    h = h[1:]

    seq = []
    for line in file:
        line = line[:-1] # remove newline

        if line[0] == '>':
            yield FastaRecord(h,''.join(seq))

            h = line[1:]
            seq = []
            continue

        #seq += [line]
        seq.append(line)
```

```
    yield FastaRecord(h,''.join(seq))


def _fasta_itr_from_name(fname):
    "Provide an iteration through the fasta records in the file named fname. "

    f = myopen(fname)
    for rec in _fasta_itr_from_file(f) :
        yield rec


def _fasta_itr(src):
    """Provide an iteration through the fasta records in file `src'.

    Here `src' can be either a file object or the name of a file.
    """
    if type(src) == str :
        return _fasta_itr_from_name(src)
    elif type(src) == file :
        return _fasta_itr_from_file(src)
    else:
        raise TypeError

def fasta_get_by_name(itr,name):
    "Return the record in itr with the given name."
    x = name.strip()
    for rec in itr:
        if rec.header.strip() == x:
            return rec
    return None
```

```
class fasta_itr (object) :
    "An iterator through a sequence of fasta records."

    def __init__(self, src) :
        "Create an iterator through the records in src."

        self.__itr = _fasta_itr(src)

    def __iter__(self) :

        return self

    def next(self) :

        return self.__itr.next()

    def __getitem__(self,name) :

        return fasta_get_by_name(iter(self),name)

    def QN_range(self) :

        # this def sreates two lists. One is header_list that holds
        # the headers from a fasta file. The other is protein_list
        # that holds all of the sequences from a fasta file. It calls
        # the QN subroutine for every value in QN_content.

        QN_content = [2,3,4,5,6]

        self.header_list = []
        self.protein_list = []
```

```
    for rec in self.__itr :

        self.header_list.append(rec.header.translate(None,','))
        self.protein_list.append(rec.sequence.strip('* '))


    for QN in QN_content :
        self.content = QN
        self.QN()

def QN(self) :

    # QN uses a recursive approach to find QN rich domains. It
    # loops through an element of self.protein_list until it gets
    # to a ten residue window that has QN content greater than
    # the entry in self.content. then it invokes the domain_check
    # routine which asks if the next 10 residue segment has QN
    # content greater than self.content. Eventually it returns
    # a contiguous QN rich domain.


    amino_acids = ['R', 'H', 'K', 'D', 'E', 'S', 'T', 'N', 'Q',\
                   'C', 'G', 'P', 'Y', 'F', 'W', 'A', 'I', 'V', 'L', 'M']

    # the output is a list that holds all output data
    # endline counter is used to keep trak of where to put the
    # endline in the output text file

    output = []
```

```
endline_counter = 0
domain_counter = 0
protein_index = -1
Q = 0
N = 0


# protein is a single protein sequence input from the fasta file
# domain is a string that holds the QN rich domain

self.domain_list = []
self.domain = ('')

for element in self.protein_list :
    protein_index += 1
    self.protein = element
    n = 0

    while n <= len(self.protein)-10 :

        # calculate the QN content of a 10 residue window

        QN = self.protein[n:n+10].count('Q') + self.protein[n:n+10].count('N')

        # if QN is greater than 40% then we start a domain

        if QN > self.content :

            # recursive function tests Q/N content and adds to the length of the domain

            self.domain_list.append(self.domain_check(n))
```

```
            for string in reversed(self.domain_list) :
                self.domain += string

            # adds to the counter so we dont reiterate Q/N rich domains

            n = n + len(self.domain)

            # check length of domain, enter domain and other data into output list

            Q = self.domain.count('Q')
            N = self.domain.count('N')

            if Q != 0 and N != 0 :
                if len(self.domain) >= 40 and (Q/N <= 4) and (N/Q <= 4)  :

                    # old code
                    # N >= ((len(self.domain)*self.content)/50) and Q >=
((len(self.domain)*self.content)/50)

                    # print (N), (Q), (Q/N), (len(self.domain)), (self.content)
                    # print (self.domain)

                    domain_counter += 1

                    output.append(self.header_list[protein_index])
                    output.append(self.domain)
                    output.append(len(self.domain))
                    output.append(n-len(self.domain))
                    output.append(n)
```

```
                for residue in (amino_acids) :
                    output.append(self.domain.count(residue))
                self.domain = ('')
                self.domain_list = []

            self.domain = ('')
            self.domain_list = []

        self.domain = ('')
        self.domain_list = []

    else : n += 1

# create output file

file_handle = open ('yeast prions2 (one fifth)"%s".txt' % (self.content), 'w')
file_handle.write (" total number of domains =")
file_handle.write (str(domain_counter))
file_handle.write ("\n")
file_handle.write ("Protein Description, Domain, Length, start residue, end residue,\
R, H, K, D, E, S, T, N, Q, C, G, P, Y, F, W, A, I, V, L, M \n")

for element in output :
    endline_counter += 1
    file_handle.write (str(element))
    file_handle.write (", ")
    if endline_counter % 25 == 0 :
        file_handle.write (" \n ")

def domain_check(self, n) :
```

```
# Recursive subroutine that searches for QN rich domains

QN = self.protein[n+10:n+20].count('Q') + self.protein[n+10:n+20].count('N')

print self.protein[n:n+10]
print QN

if (QN) >= self.content :

    self.domain_list.append(self.domain_check(n+10))

return self.protein[n:n+1]
```