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Amyloid Diversity, Translation, and the Yeast Prion [*PSI*$^+$]

by

Catherine Kuan-Chi Foo

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

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by
Catherine Kuan-Chi Foo

for Aaron and Gus

Acknowledgments

The major components of many scientific trajectories are luck, opportunity, and the kindness of other scientists who agree to be your mentor for little or nothing in exchange. As this chapter of my scientific life draws to a close, I have many of these people to thank.

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I joined the Weissman lab because I was impressed by the graduate students and I wanted to learn from them. Years later they are some of the most important people in my life, both inside and outside the lab. In particular, the [PSI^+] subgroup has defined my graduate school experience. Sean Collins was my rotation mentor and one of the smartest guys I know. I would like to publicly apologize for once telling him that he was a terrible rotation mentor. Kim Tipton guided me through my early years in the lab and has continued to be a frequent source of advice. I joined the lab because I wanted to be more like her – very smart, very talented, with a true passion for science. Clement Chu is kind to a fault. He has fielded many texts, phone calls, and late-night emails from me. These either

involve me desperately needing help with lab equipment, or are about food. I hope he leaves the lab soon. I am beyond indebted to Brandon Toyama, who taught me HXNMR and patiently answered every dumb question that I asked him. He has a quiet sense of humor that I enjoy, and it was a huge loss to me when he graduated. Katie Verges always had a smile and an enthusiastic answer to any question that I asked. Dale Cameron was so positive about everything and was a great teacher. Melanie Smith is an incredibly careful scientist and my favorite rotation student. I was really glad when she joined our lab.

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C.K.F.
San Francisco
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The text of this dissertation is set in Monotype Garamond. This typeface was designed in 1922 by typography legend Stanley Morison, who also brought us the ubiquitous fonts Times and Times New Roman. Monotype Garamond is based on roman types cut by Jean Jannon in the early seventeenth century after the designs of Claude Garamond (c. 1480-1561). Jannon's typeface was "rediscovered" in 1825, and misattributed to Garamond until its true origin was discovered by type historian[1] Beatrice Warde in the late 1920s. Garamond is considered to be among the most readable serif typefaces in offline print – and is also the favorite font of Aaron Bastian.

[1] An alternate career of which I was not previously aware.

Amyloid Diversity, Translation, and the Yeast Prion [PSI^+]

Catherine Foo

Abstract

Yeast prions have proved to be an excellent model for learning about the biophysical properties of prions. In particular, the [PSI^+] prion has been invaluable for elucidating the structural basis of prion strains and the prion species barrier. The [PSI^+] prion itself is an aggregated form of the translation termination factor Sup35. The aggregation of Sup35 into beta-sheet rich amyloid fibers reduces its activity levels, resulting in translational readthrough of stop codons. In this dissertation, I explore the diversity of amyloid structures formed by a chimeric Sup35 protein, and describe my work to investigate the translational effects of the [PSI^+] prion.

In the first section, I describe my work examining the amyloid structure of a chimeric yeast prion that is able to form two species-specific conformations. These conformations were probed by hydrogen/deuterium exchange and nuclear magnetic resonance, a technique that has been successfully applied to the study of many amyloids. I discovered that these two conformations were radically different: the residues that comprised the "amyloid core" of each conformation was entirely distinct from the other. In addition to emphasizing the diversity of amyloid formation, this result also reveals that amyloid formation in one region of a polypeptide can preclude the formation of amyloid in another amyloidogenic sequence in the same polypeptide.

In the second section, I describe an investigation into the translational effects of the yeast prion [PSI^+] by using a technique called ribosome profiling. Ribosome profiling takes

advantage of next-generation sequencing technologies to deeply sequence the short fragments that are protected by ribosomes. We can examine the differences in translation in [*PSI*$^{+}$] and isogenic [*psi*$^{-}$] yeast strains to uncover the regulatory effects of this yeast prion.

Contributions

Chapter 3, "Radically different amyloid conformations dictate the seeding specificity of a chimeric Sup35 prion," originally appeared as a manuscript published in the *Journal of Molecular Biology*, Vol. 408, pp. 1-8. Dr. Motomasa Tanaka and Dr. Yumiko Ohhashi performed the limited proteolysis and mass spectrometry experiments. Dr. Mark J.S. Kelly assisted in setting up NMR experiments and NMR data analysis. Chapter 4, "Uncovering the translational effects of [*PSI*$^+$] with ribosome profiling," describes a collaboration with the laboratory of Susan Lindquist at the Whitehead Institute. Work performed in her lab by Randal Halfmann, Dan Jarosz, and Alex Lancaster has been clearly attributed. In Appendix A, "Development of a fluorescence-tagged Sup35," the alignments to Sup35 homologs was done by Dr. Kimberly A. Tipton. Dr. Tipton also helped develop the overall strategy.

With the exception of those items listed above, the work presented in this dissertation was performed by its author, Catherine Foo, under the supervision of Dr. Jonathan S. Weissman.

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Chapter I

Introduction

Background

Prions came to the forefront of public imagination in the late 90s, when cases of the fast-progressing neurodegenerative disease variant Creutzfeldt-Jacob Disease (vCJD, commonly called "mad cow disease") appeared to be linked to the consumption of contaminated beef (Collinge *et al.* 1996; Will *et al.* 1996; Bruce *et al.* 1997; Hill *et al.* 1997). However, the work that unveiled the nature of the prion had started many decades earlier with the study of scrapie, a prion disease of sheep (see Prusiner 1998). We now know that prions are caused by the toxic misfolding and aggregation of a native protein, PrP. To distinguish the prion and native states, they are referred to as PrP^{Sc} and PrP^{C} respectively.

Prions differ from other infectious agents in that they are composed solely of proteins with no foreign nucleic acid (reviewed in Caughey and Baron 2006). Therefore, all information about propagation of the prion must be encoded within the protein itself. This has led to much research to uncover the molecular mechanisms behind phenomena such as the existence of prion strains (reviewed in Collinge and Clarke 2007). Prion strains are analogous to strains in bacterial or viral diseases in that disease symptoms can vary among strains. However, unlike bacterial or viral diseases, there exists no nucleic acid in which to encode these differences. In addition, prion strains can be observed for prions that are formed from PrP proteins that have identical amino acid sequences (reviewed in Aguzzi *et al.* 2007, Morales *et al.* 2007, and Sweeting *et al.* 2010).

An infectious agent composed solely of proteins must also have a mechanism to replicate once it enters the host. Unlike conventional infections, where a bacteria or virus replicates itself by hijacking the host's resources, a prion particle must convert endogenous copies of PrP^{C} to the pathogenic PrP^{Sc} prion form. The interactions between the foreign

PrP^{Sc} and the endogenous PrP^{C} is a major factor in prion infection and is also one of the determinants for the interspecies prion transmission barrier, commonly referred to as the "species barrier" (Collinge and Clarke 2007).

The prion concept has been generalized to describe a class of phenomena in yeast, the most well-studied of which is [*PSI*$^+$]. These yeast prions, although not pathogenic, have been invaluable in uncovering many of the biophysical aspects of mammalian prions. In particular, our ability to generate synthetic prions *in vitro* and demonstrate their infectivity (Sparrer *et al.* 2000; King and Diaz-Avalos 2004; Tanaka *et al.* 2004; Tanaka and Weissman 2006) has allowed us to demonstrate definitively the protein-only nature of prions and to uncover the structural basis of other prion phenomena such as prion strains and the interspecies transmission barrier (Chien *et al.* 2004; Jones and Surewicz 2005; Tanaka *et al.* 2005; Tanaka *et al.* 2006; Tessier and Lindquist 2007; Toyama *et al.* 2007; Chang *et al.* 2008; Verges *et al.* 2011; reviewed in Cobb and Surewicz 2007 and Tessier and Lindquist 2009).

[*PSI*$^+$] is a prion of the translation termination factor Sup35. Originally identified in 1965 (Cox 1965), the molecular nature of [*PSI*$^+$] was unclear (Cox *et al.* 1988) until Reed Wicker proposed that [*PSI*$^+$] and another yeast prion [URE3] shared many of the characteristics that defined prions in mammals (Wickner 1994; Weissmann 1994; Wickner *et al.* 1995).

In normal cells without the [*PSI*$^+$] prion (referred to as [*psi*$^-$] cells), Sup35 exists as soluble monomers. These Sup35p monomers interact with the ribosome to recognize stop codons. In [*PSI*$^+$] cells, however, Sup35 is aggregated into amyloid fibers, which reduces their ability to interact with ribosomes. These fibers grow by recruiting monomeric Sup35, and divide via a chaperone-mediated process (Patino *et al.* 1996; reviewed in Tuite and Cox

2003 and True 2006) which both increases the number of fibers and decreases their size. Some of these fibers are partitioned to the daughter bud during cell division, allowing [*PSI*$^+$] to be maintained stably.

Strains of [*PSI*$^+$] are encoded by amyloid conformations

The presence of [*PSI*$^+$] in a yeast cell can be detected by using a color reporter based on a mutation in the adenine biosynthesis pathway. A premature stop codon in either *ADE1* (*ade1-14*) or *ADE2* (*ade2-1*) results in the accumulation of a metabolic intermediate that causes cells to appear red. In the presence of [*PSI*$^+$], however, translational readthrough allows full length Ade1 or Ade2 protein to be produced, restoring the pathway and returning the cells to their original white color.

Prion strains, or variants, of [*PSI*$^+$] can be observed using this reporter. When the cell appears white, the [*PSI*$^+$] strain is referred to as a "strong" strain. Some [*PSI*$^+$] strains will result in a pink rather than white color, due to a smaller degree of translational readthrough, and are referred to as "weak" strains. A series of experiments simultaneously proved the protein-only hypothesis for yeast prions and demonstrated that distinct, heritable prion strains could be obtained by altering the amyloid conformation of Sup35 fibers (Tanaka *et al.* 2004). The distinct amyloid conformations were formed *in vitro* by polymerizing the fibers at either 4°C (resulting in the Sc4 conformation) or 37°C (resulting in the Sc37 conformation). These fibers were also studied by hydrogen-deuterium exchange and nuclear magnetic resonance (HXNMR) (Hoshino *et al.* 2002; Kuwata *et al.* 2003; Yamaguchi *et al.* 2004; Carulla *et al.* 2005; Lührs *et al.* 2005; Ritter *et al.* 2005; Olofsson *et al.* 2006; Olofsson *et al.* 2007; Wilson *et al.* 2007; Morgan *et al.* 2008; Vilar *et al.* 2008; Carulla *et*

al. 2009; Olofsson *et al.* 2009; Wasmer *et al.* 2010; Cho *et al.* 2011; Konuma *et al.* 2011), revealing differences in the amyloid structure of these fibers that explained observed differences between the Sc4 and Sc37 fibers in terms of propensity to grow and divide (Toyama *et al.* 2007).

[PSI^+] and the prion species barrier

The yeast prion [PSI^+] can also be used to study the prion species barrier. Species barriers are found between most yeast species (Chernoff *et al.* 2000; Kushnirov *et al.* 2000; Santoso *et al.* 2000; Nakayashiki *et al.* 2001; Resende *et al.* 2002; Chien et al. 2004; Chen *et al.* 2007; Vishveshwara and Liebman 2009; Chen *et al.* 2010; Afanasieva *et al.* 2011). In addition, many of these barriers are exquisitely sensitive to the prion strain. To study the species barrier *in vivo*, Sup35 from various yeast species can be expressed in *S. cerevisiae*. Analogous experiments can be performed *in vitro* with using fiber seeding assays.

Observations regarding the species barrier and [*PSI*+] have reflected many of the puzzling observations in mammalian prion disease. For example, a prion that crosses the species barrier is often accompanied by a change in disease phenotype (Mahal *et al.* 2010). Similarly, in the [PSI^+] model, Sc4 (a prion composed of *S. cerevisiae* Sup35 with a strong phenotype, described above) can occasionally be induced to cross the species barrier to *Candida albicans*. However, upon crossing back into the *S. cerevisiae* context, the phenotype is greatly weakened (Tanaka *et al.* 2005).

Why do yeast have prions?

Why do yeast cells contain [PSI^+] and other prions? The conservation of the Sup35 prion domain across yeast species suggests that selective forces have favored the preservation of the ability to form prions. Many of the proteins that have prion-forming domains are transcription factors or RNA-binding proteins, suggesting that prions may act as regulatory switches for these pathways (Du *et al.* 2008; Alberti *et al.* 2009).

In addition, Susan Lindquist has proposed that the increase of translational readthrough in [PSI^+] cells allows the unmasking of genetic variation that has accumulated in non-coding sequences (True and Lindquist 2000; True *et al.* 2004; Giacomelli *et al.* 2007). Thus, [PSI^+] behaves as a switch that can reveal the effects of multiple mutations at once, similar to the ability of a capacitor to store and release accumulated electric charge in a circuit. This "evolutionary capacitor" could increase the phenotypic diversity of a population of yeast cells during times of stress.

The hypothesis that prions have a function is controversial (Edskes *et al.* 2009; Wickner *et al.* 2010). Regardless of whether prions have an intended function, the ability of Sup35 to adopt a prion form has consequences on the evolution of *S. cerevisiae* and other budding yeasts. Acquiring a detailed understanding of the translational effects of [PSI^+] may provide some insight into these questions.

Summary

In this thesis, I explore both structural and functional aspects of the yeast prion [PSI^+]. Chapter 2 provides a literature review on a particular fusion of the *S. cerevisiae* and *C. albicans* prion domains ("Chimera") that has revealed many aspects of the prion species

barrier. Chapter 3, a manuscript published in the *Journal of Molecular Biology*, details the investigation of two amyloid conformations of Chimera by HXNMR. In Chapter 4, I describe a project in progress to identify the effects of [*PSI*$^{+}$] on translation termination by using ribosome profiling. Chapter 5 provides a brief conclusion. In Appendix A, I briefly describe the creation of a fluorescence-tagged Sup35 construct.

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Chapter II

A review of Chimera literature

Introduction

The interspecies prion transmission barrier, commonly referred to as the "species barrier," inhibits the transmission of prions from one species to a host of another species. In the most infamous example, the transmission of bovine spongiform encephalopathy (BSE), or "mad cow disease," from cow to human is due to the ability of BSE to cross this species barrier (Collinge *et al.* 1996; Will *et al.* 1996; Bruce *et al.* 1997; Hill *et al.* 1997; see also Bruce *et al.* 1994). Given its medical relevance, understanding how species barriers modulate prion transmission is critical.

Although the yeast prion [*PSI*$^+$] is not pathogenic, it has been invaluable for understanding the molecular and structural basis for the species barrier. In this section, I will briefly summarize methods for studying the species barrier in [*PSI*$^+$] and review the historical underpinnings for the work described in Chapter 3.

Studying the species barrier in [*PSI*$^+$]

The yeast prion [*PSI*$^+$], which is caused by the aggregation of the Sup35 translation termination factor, can be used to model the species barrier both *in vivo* and *in vitro*. To study the species barrier *in vivo*, the Sup35 homolog from a foreign yeast species can be expressed in a [*PSI*$^+$] cell. By monitoring [*PSI*$^+$]-dependent readthrough using a color reporter (a mutation in the adenine biosynthesis pathway which causes the accumulation of a red pigment), one can determine whether the foreign Sup35 can propagate the particular [*PSI*$^+$] prion strain that is present. One can also engineer a strain of *Saccharomyces cerevisiae* to express a prion composed solely of foreign Sup35 and thereby test the species barrier between any two species of Sup35, or in fact, any genetic variant of Sup35 (Chernoff *et al.* 2000;

Kushnirov *et al.* 2000; Santoso *et al.* 2000; Nakayashiki *et al.* 2001; Resende *et al.* 2002; Chien et al. 2004; Chen *et al.* 2007; Vishveshwara and Liebman 2009; Chen *et al.* 2010; Afanasieva *et al.* 2011).

In vitro, the species barrier can be investigated with a polymerization assay in the presence of a dye that binds amyloid such as Congo Red or thioflavin T. Preformed, sonicated amyloid fibers of Sup35 can be incubated with foreign Sup35 monomers, and the ability of the polymerization of a foreign monomer to be "seeded" by the preformed fibers can be measured by monitoring the initial rate of increase in fluorescence.

Historical origins of Chimera

The Sup35 fusion ("Chimera") that is described in Chapter 3 consists of the first 40 residues of *S. cerevisiae* Sup35 followed by residues 47-141 from *Candida albicans* Sup35. This fusion was created in a series of experiments designed to identify the region of *S. cerevisiae* Sup35 that was important for the self-recognition of Sup35 (Santoso et al. 2000). In this work, different regions of *S. cerevisiae* Sup35 were replaced with the corresponding sequences of *C. albicans* Sup35, and these fusions were expressed in [PSI^+] cells. In an assay similar to one described earlier (DePace *et al.* 1998), these fusions were screened for their ability to propagate the [PSI^+] prion. This screen identified Chimera as containing residues essential for the "self-recognition" of *S. cerevisiae* Sup35.

Surprisingly, this Chimera was also able to interact with *C. albicans* Sup35 (Chien and Weissman 2001). Naively one might assume that the location of the region of self-identification would be conserved from one species to the next, but it appeared that Chimera also contained the appropriate region for *C. albicans* recognition.

Chimera forms two strains

Phenotypic and biochemical evidence suggested that Chimera was able to form two strains, one, Chim[SC], that was compatible with *S. cerevisiae* prions, and a second, Chim[CA], that was compatible with C. albicans prions (Chien and Weissman 2001). The two strains had distinct color phenotypes: Chim[SC] appeared to be a weaker strain (pink) and Chim[CA] appeared to be stronger (white). When digested with chymotrypsin, the two forms of Chimera revealed very different digestion patterns. *In vitro* evidence also pointed to the existence of two strains. Chim[SC] amyloid fibers formed in vitro displayed a strong preference for seeding polymerization of *S. cerevisiae* Sup35 monomers, while Chim[CA] displayed a corresponding preference for *C. albicans* monomers.

In a subsequent paper, Peter Chien also described mutations and polymerization conditions that would cause Chimera to favor the formation of one conformation over the other (Chien *et al.* 2003). For example, polymerization at low temperatures favored the Chim[SC] conformation, and polymerization at high temperatures favored the Chim[CA] conformation. In addition, specific mutations altered the propensity of Chimera to form each of these conformations. Mutations Q15R and S17R are both located in the SC-derived (*S. cerevisiae*-derived) sequence of Chimera and are known to impair polymerization of *S. cerevisiae* Sup35 (DePace et al. 1998). When these mutations were introduced into the Chimera sequence, Chimera was no longer able to form the Chim[SC] conformation. Similarly, a series of mutations in the CA-derived region (G64A, G65A, G74A, G75A; coordinates are relative to the Chimera sequence and the four mutants are collectively

referred to as GA4[1]) was able to promote formation of Chim[SC]. In addition to revealing the relationship between point mutations, strains, and species barriers, this work also strongly suggested that the amyloid conformations of Chim[SC] and Chim[CA] were structurally different.

Chimera amyloids can be studied *in vitro*

When formed *in vitro*, these amyloids have also been demonstrated to be infectious prions with distinct species specificity (Tanaka *et al.* 2005). Chim[SC] and Chim[CA] were transformed into yeast expressing *S. cerevisiae*, *C. albicans*, or Chimera Sup35 alleles at the endogenous Sup35 locus. Both strains could "infect" Chimera-expressing yeast, but Chim[SC] could only induce prion formation in yeast expressing *S. cerevisiae* Sup35, and Chim[CA] could only induce prion formation in yeast expressing *C. albicans* Sup35. In addition, the same *in vivo* color phenotypes observed by Chien and Weissman (Chien and Weissman 2001) were also observed here.

Using a very different assay, the Lindquist laboratory also demonstrated that the species specificity of Chimera could be reproduced *in vitro* (Tessier and Lindquist 2007). Peptide arrays were used to identify sequences that could nucleate the polymerization of Sup35 variants including Chimera. Consistent with previous observations, Chimera preferentially nucleated from species-specific peptides depending on polymerization conditions.

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[1] In the context of the *C. albicans* Sup35 prion domain, all four mutations were required to abolish amyloid formation. Chien also tested mutations at other locations that did not affect the ability of *C. albicans* Sup35 to polymerize (refer to Peter Chien's lab notebook "CA PrD GG->AA 2000").

Having established that Chimera was able to form two distinct strains, we decided to investigate the amyloid structure of Chimera in its two strains with hydrogen-deuterium exchange and NMR (Hoshino *et al.* 2002; Kuwata *et al.* 2003; Yamaguchi *et al.* 2004; Carulla *et al.* 2005; Lührs *et al.* 2005; Ritter *et al.* 2005; Olofsson *et al.* 2006; Olofsson *et al.* 2007; Wilson *et al.* 2007; Morgan *et al.* 2008; Vilar *et al.* 2008; Carulla *et al.* 2009; Olofsson *et al.* 2009; Wasmer *et al.* 2010; Cho *et al.* 2011; Konuma *et al.* 2011). This work is described in the Chapter 3, which is a manuscript published in the *Journal of Molecular Biology*.

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Chapter III

Radically different amyloid conformations dictate the seeding specificity of a chimeric Sup35 prion

Radically different amyloid conformations dictate the seeding specificity of a chimeric Sup35 prion

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Abstract

A remarkable feature of prion biology is that the same prion protein can misfold into more than one infectious conformation and these conformations, in turn, can lead to distinct heritable prion strains with different phenotypes. The yeast prion [PSI^+] has emerged as a powerful system for studying how changes in the strain conformation affect cross-species transmission. We have previously established that a chimera of the *Saccharomyces cerevisiae* (SC) and *Candida albicans* (CA) Sup35 prion domains can cross the SC/CA species barrier in a strain-dependent manner. *In vitro*, the conversion of the monomeric chimera to the prion (amyloid) form can be seeded by either SC or CA Sup35 amyloid fibers, resulting in two strains: Chim[SC] and Chim[CA]. These strains have a "molecular memory" of their originating species in that Chim[SC] preferentially seeds conversion of SC Sup35, and vice versa. To investigate how this species specificity is conformationally encoded, we used amide exchange and limited proteolysis to probe the structures of these two strains. We find that the amyloid cores of Chim[SC] and Chim[CA] are predominantly confined to the SC- and CA-derived residues respectively. In addition, the chimera is able to propagate the Chim[CA] conformation even when the SC residues that comprise the Chim[SC] core were deleted. Thus the two strains have non-overlapping and modular amyloid cores that determine whether SC or CA residues are presented on the growing face of the prion seed. These observations establish how conformations determine the specificity of prion transmission and demonstrate the remarkable plasticity of amyloid misfolding.

Introduction

Prions, originally postulated to explain transmissible spongiform encephalopathies (Prusiner 1998), also underlie a number of epigenetic elements in fungi and perhaps in higher organisms (Shorter and Lindquist 2005). Arguably the best studied of these elements is the yeast prion [*PSI*$^+$], which results from self-propagating aggregates of the Sup35 translation termination factor (Wickner 1994). Although Sup35 and the mammalian prion protein PrP have unrelated amino acid sequences, both proteins misfold into ordered, beta-sheet rich amyloid fibers. The self-templating nature of amyloid fibers allows the prion state to be stably propagated through the continual binding and conversion of newly-synthesized soluble proteins to the prion form (Tuite and Cox 2003; Chiti and Dobson 2006; Caughey *et al.* 2009; Greenwald and Riek 2010). Sup35 amyloid fibers, when introduced into yeast cells via protein transformation, induce cells to convert to the [*PSI*$^+$] state with high efficiency (Sparrer *et al.* 2000; King and Diaz-Avalos 2004; Tanaka *et al.* 2004). These studies provided direct demonstration of the "protein-only" prion hypothesis and established amyloid as the infectious form of the Sup35 protein.

Remarkably, a single prion protein can adopt a spectrum of amyloid conformations that lead to heritable strain variants (Derkatch *et al.* 1996; Collinge and Clarke 2007). These strain variants manifest as distinct pathological symptoms in mammalian prions and as differences in the strength and stability of heritable phenotypes in yeast prions including [*PSI*$^+$]. Beyond causing phenotypic differences, strains can also have different propensities for crossing "species barriers" (Chien and Weissman 2001; Chien *et al.* 2003; Tanaka *et al.* 2005; Collinge and Clarke 2007), which inhibit the transmission of prions between species, even those with closely related prion proteins (Chernoff *et al.* 2000; Kushnirov *et al.* 2000;

Santoso *et al.* 2000). The central relationship between strains and species barriers has been underscored by "new variant" Creutzfeld-Jacob disease ("mad cow disease"), which is attributed to a strain of bovine spongiform encephalopathy with an enhanced ability to cross the species barrier to humans (Bruce *et al.* 1997; Hill *et al.* 1997; Collinge and Clarke 2007). In the case of [PSI^+], distinct conformations of Sup35 can be formed *in vitro* by altering conditions of polymerization, such as temperature. When introduced into yeast, these conformations can induce different prion strains, establishing that heritable differences in prion strain variants are enciphered within the conformation of the infectious protein (King and Diaz-Avalos 2004; Tanaka et al. 2004). The ability to relate the physical properties of synthetic prions to their biological effects provides a critical tool for exploring basic principles of prion inheritance, including how changes in a prion's conformation alter its ability to template or "seed" the polymerization of Sup35 from other species. As a result, [PSI^+] has been particularly valuable for exploring the relationship between prion strains and species barriers (Chien and Weissman 2001; Chien et al. 2003; Tessier and Lindquist 2007).

Previously we found that a chimeric Sup35 (Chimera; **Fig. 1a**) constructed by replacing residues 41-123 of *Saccharomyces cerevisiae* (SC) Sup35 with the corresponding residues 47-141 from *Candida albicans* (CA) could cross the SC/CA species barrier in a strain-dependent manner (Chien and Weissman 2001; Chien et al. 2003). Even though Chimera was originally created to identify a minimal region of SC Sup35 required for self-recognition (Santoso et al. 2000), the conversion of Chimera to the prion form could be seeded by both SC and CA Sup35. When seeded by SC Sup35, Chimera forms a strain we call Chim[SC] and this strain readily seeds SC but not CA Sup35 (**Fig. 1b,c**). In contrast, seeding Chimera with CA Sup35 results in the Chim[CA] strain, which preferentially seeds conversion of CA

Sup35. Thus, the Chim[SC] and Chim[CA] strains retain a molecular memory of their seed. Elegant peptide array experiments by Tessier and Lindquist revealed that short peptides of Sup35 could drive polymerization of Chimera into the amyloid form (Tessier and Lindquist 2007). Chimera preferentially interacted with peptides derived from SC or CA Sup35 at temperatures (Chien et al. 2003) that favored Chim[SC] or Chim[CA] polymerization, respectively. The above studies established that the conformation of each Chimera prion form dictates seeding specificity, presumably by presenting different regions of the protein on the growing amyloid face. Nonetheless, the nature and extent of these conformational differences and how they alter seeding specificity remain largely unexplored.

Hydrogen exchange reveals reciprocal regions of protection

To address these questions, we used amide hydrogen/deuterium exchange (HX) coupled to multidimensional NMR (Hoshino *et al.* 2002; Toyama *et al.* 2007) to probe the conformations of Chim[SC] and Chim[CA]. HX NMR can provide atomic-level information about which residues are involved in stable hydrogen bonds, including those that comprise the structural core of amyloid fibers. Here, uniformly ^{15}N-labeled fibers are placed in a D_2O-containing buffer to allow exchange of solvent-accessible hydrogens. After quenching, the fibers are dissolved in DMSO to a monomeric form amenable to the collection of high-resolution spectra. Exchange of the backbone amide hydrogen of a given residue results in a decrease in the signal of the corresponding peak in the 2D ^{15}N-HSQC spectrum.

Analysis of the data requires assignment of the spectrum of Chimera, which is challenging due to the extensive glutamine stretches and multiple sequence repeats in the CA

domain. By transferring existing assignments from SC Sup35 (Toyama et al. 2007) and assigning additional peaks with seven three-dimensional NMR experiments on uniformly ^{13}C-,^{15}N-labeled Chimera, we succeeded in assigning 142 residues (**Fig. 1d**). This includes 30 of the first 40 SC-derived residues (Ch1-40) and 33 of the CA-derived residues (Ch41-135). With these assignments, we were able to monitor protection from exchange at many residues throughout the prion domain on Chim[SC] and Chim[CA] fibers after 1 minute, 1 hour, and 1 day of exchange.

The regions most protected from exchange in Chim[SC] and Chim[CA] were nearly perfectly reciprocal (**Figs. 2** and **3a**). The residues most strongly protected in Chim[SC] were those derived from the SC Sup35 prion domain (Ch1-40), while the residues most strongly protected in Chim[CA] were CA-derived. This immediately suggested that the amyloid cores of Chim[SC] and Chim[CA] were radically different. Furthermore, the protected region in Chim[SC] resembled that of the seeding SC conformation (see legend to **Fig. 1a**) which has a compact core limited to the first 40 residues (Toyama et al. 2007). Consistent with previous studies (Tanaka et al. 2004; Krishnan and Lindquist 2005; Toyama et al. 2007), the Sup35 middle domain, Ch136-265, which is not essential for prion behavior (Liu *et al.* 2002), showed minimal protection from exchange indicating that it was easily solvent-accessible and thus relatively unstructured (**Fig. S1**).

Limited proteolysis confirms differences in amyloid cores

We also used limited proteolysis to identify the protease-resistant core (Sajnani *et al.* 2008) of Chim[SC] and Chim[CA]. Long protected peptides that persist during digestion with proteinase K were found to be a reliable method for distinguishing distinct amyloid

strain conformations of SC Sup35 (M.T., unpublished observations). Chim[SC] and Chim[CA] were digested, pelleted by ultracentrifugation, and dissolved in DMSO. The resulting peptides were identified by MALDI-TOF mass spectrometry. Consistent with our amide exchange results, the only long peptides observed in Chim[SC] following proteinase K treatment spanned residues 2-45 and 2-49 (**Figs. 3b** and **S2**). In contrast, digestion of Chim[CA] revealed long protected peptides derived from residues 50-131 in addition to peptides spanning residues 2-49.

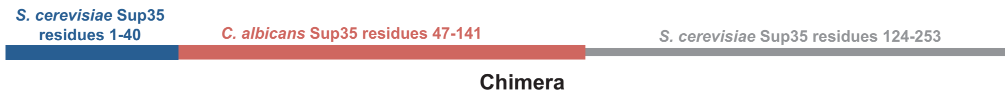
Proteolysis confirmed the differences observed by HX in the protected regions of Chim[SC] and Chim[CA], but both conformations had protease-resistant peptides spanning Ch1-40. This protease protection could indicate that Chim[CA] has an expanded amyloid core that comprises almost the entire prion domain. Alternatively, these residues could be protected from proteolysis due to other factors such as secondary aggregation or partial structure while not being essential for the structural integrity of the amyloid core.

Chim[CA] does not require SC-derived residues 1-40

To test whether the protection observed in Ch1-40 was structurally critical for the Chim[CA] core, we created a mutant Chimera without these SC-derived residues (ChΔSc). ChΔSc retained the ability to polymerize into an amyloid form and this polymerization was efficiently seeded by CA and Chim[CA] but not by SC or Chim[SC] (**Figs. 4a-c** and **4d-f**). Moreover, when seeded by CA, the resulting amyloid conformation (ChΔSc[CA]) exhibited the same species specificity as Chim[CA] (**Fig. 4g,h**). Taken together, the above studies argue that the minimal structural core required for propagation of the Chim[CA] conformation is composed solely of CA residues and is thus distinct from that of Chim[SC].

Figure 1

a



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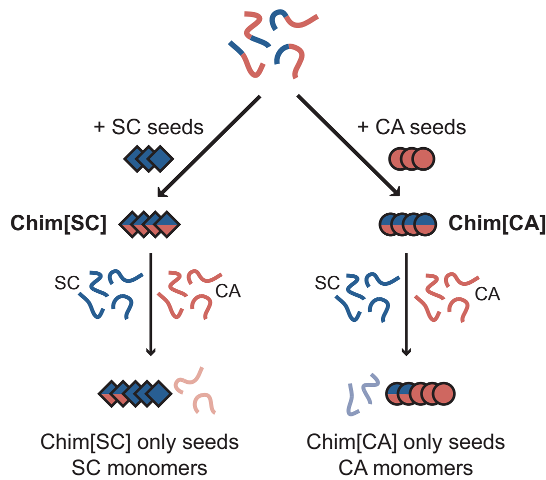
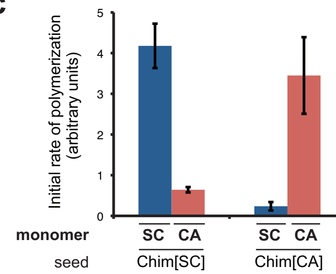


c



d

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1   MSDSNQGNNQ QNYQQYSQNG NQQQGNNRYQ GYQAYNAQAQ SFVPQGGYQQ
51  FQQFQPQQQQ QQYGGYNQYN QYQGGYQQNY NNRGGYQQGY NNRGGYQQNY
101 NNRGGYQGYN QNQQYGGYQQ YNSQPQQQQQ QQSQGMSLND FQKQQKQAAP
151 KPKKTLKLVS SSGIKLANAT KKVGTKPAES DKKEEEKSAE TKEPTKEPTK
201 VEEPVKKEEK PVQTEEKTEE KSELPKVEDL KISESTHNTN NANVTSADAL
251 IKEQEEEVDD EVVNDHHHHH HHHH
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Figure 1. Chimera sequence and assignments.

(**a**) Schematic diagram of Chimera. Chimera and all other proteins in this study are expressed as the prion domain indicated followed by the SC Sup35 middle domain and a C-terminal 7xhistidine or 9xhistidine tag not shown in the schematic (Santoso et al. 2000; Chien and Weissman 2001). Proteins were purified as described (DePace *et al.* 1998). These proteins do not include the Sup35 C-terminal domain that confers the translation termination function because it is not required for amyloid formation *in vitro*. (**b**) Cartoon representation of the seeding specficity of Chim[SC] and Chim[CA]. (**c**) Species specificity of Chim[SC] and Chim[CA]. Amyloid fibers were formed and assayed for species specificity as previously described (Chien et al. 2003). Chim[SC] and Chim[CA] were formed by two rounds of Chimera polymerization, with 5% seeding (w/w) by preformed SC or CA fiber seeds as indicated. Resulting fibers contain no more than 0.25% of SC or CA seed. To take advantage of previous work characterizing structural features of defined SC strains (Toyama et al. 2007), Chim[SC] was seeded by SC fibers formed at 4°C. Chim[CA] was formed at 37°C to optimize stability of species specificity. Error bars represent s.e.m. for 3 or 5 replicates. (**d**) Chimera assignments. SC Sup35 residues 1-40 are red; CA 47-141 are blue; SC 124-253 are black. Exact repeats of 5 or more residues are underlined, except the two polyglutamine repeats at residues 57-62 and 126-132. Green highlighting indicates assigned residues with distinct NMR peaks, blue indicates assigned residues that exactly overlap another peak, and yellow indicates assigned residues that are in crowded areas and thus cannot be distinguished. G64/G116 and G89/108 could be mapped ambiguously to pairs of residues respectively but not further resolved; these residues have been highlighted in purple.

Figure 2

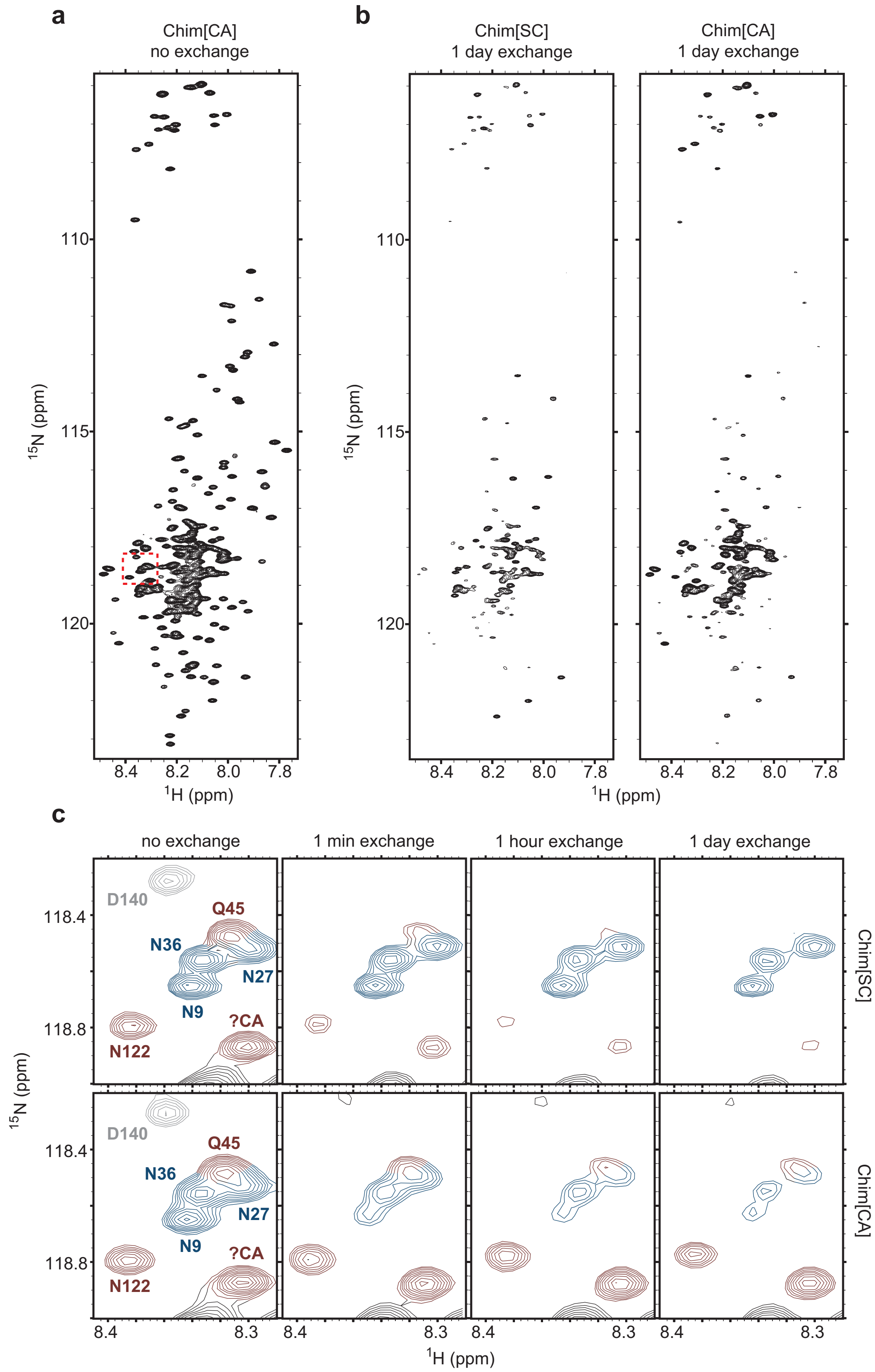

Figure 2. Hydrogen/deuterium exchange of Chimera fibers.

Hydrogen/deuterium exchange and NMR on ^{15}N-labeled Chim[SC] and Chim[CA] fibers was performed as previously described (Toyama et al. 2007) with the following modification. Rather than pelleting fibers by ultracentrifugation and resuspending fibers into D_2O-containing buffer to start the exchange, freshly prepared ^{15}N-Chimera fibers were first concentrated using an Amicon Ultra-15 centrifugal filter unit with Ultracel-30 membrane (Millipore), then diluted 12.5-fold into the equivalent buffer in D_2O at pH 7.0 to start the exchange. Time points were taken at 0 minutes, 1 minute, 1 hour, and 1 day of exchange. Exchange was quenched by adjusting pH to 2.5 with DCl. (**a**) ^{15}N-HSQC spectrum for Chim[CA], no exchange. Chim[SC] spectrum appears very similar. Red dashed box indicates residues shown in (c). (**b**) Spectra for Chim[SC] and Chim[CA], 1 day exchange. After 1 day of exchange, the same spectra for Chim[SC] and Chim[CA] qualitatively reveal large differences from the no exchange spectra and from each other. (**c**) A subset of residues (indicated by red box in (a)) from Chim[SC] and Chim[CA] after no exchange and 1 minute, 1 hour, and 1 day of exchange. Peaks are colored according to assignments: blue, Ch1-40 (SC-derived); red, Ch41-135 (CA-derived); gray, Ch136-253 (middle domain). Because most residues at the boundaries of the SC and CA segments of Chimera were assigned, a number of unassigned peaks (including the peak denoted as ?CA) could be identified as originating from CA residues due to the lack of a corresponding peak in SC spectra (see **Supplementary methods** for details on peak assignments).

Figure 3

Figure 3. Dramatically different regions of Chim[SC] and Chim[CA] are protected from amide exchange and proteolysis.

(**a**) Amide exchange data for Chim[SC] and Chim[CA] mapped to residue location. Intensities for all assigned and distinct peaks in the Chimera prion domain are plotted as a fraction of the unexchanged intensity. Estimated minimum peak intensity (dotted line) is calculated based on maximum exchange observed in the middle domain (**Fig. S1**). For overlapping peaks, values represent the combined intensities. For ambiguous peaks, intensities of both peaks are plotted. (**b**) Limited proteolysis of Chim[SC] and Chim[CA]. Chimera amyloid fibers (5 μM, 1 ml) in 5 mM potassium phosphate, 150 mM NaCl, pH 7.5 were digested with proteinase K (1.5 μg/ml) at room temperature for 2 hours. After the amyloid solution was ultracentrifuged at 214,000g for 30 min, the supernatant was removed and the pellet was washed with 1 ml of buffer and ultracentrifuged again. The pellet was dissolved in 100 μl DMSO (similar results were found with 6 M guanidine HCl, 25 mM Tris, pH 7.5). For the MALDI-TOF MS measurement, the dissolved peptides were desalted by NuTip C4 (Glygen) and analyzed with Microflex (Bruker Daltonics). As a matrix, we used 3,5-dimethoxy-4-hydroxycinnamic acid. Identification of peptides was performed using the program PAWS (ProteoMetrics). Summary of peptides identified are schematically diagrammed here.

Figure 4

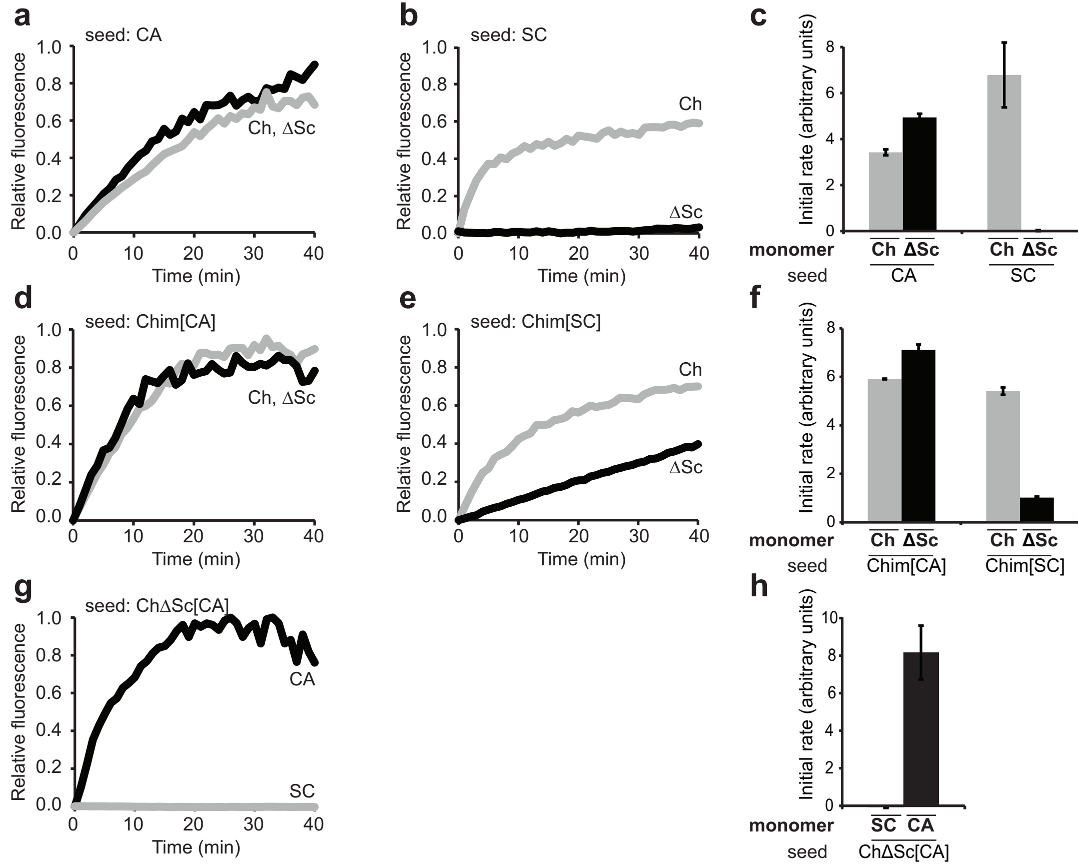

Figure 4. Chim[CA] does not require SC-derived Ch1-40 residues.

Sonicated fibers were added to monomers as indicated, and polymerization was monitored by an increase in ThioflavinT fluorescence. Data were normalized to initial and final intensities, initial time points were fit to a line, and the slope was calculated as the initial rate of polymerization. Representative normalized kinetic traces are shown. Error bars represent s.e.m. for 2-5 replicates. (**a, b, c**) Chimera and ChΔSc monomers were seeded by CA and SC fibers. ChΔSC, constructed with standard molecular cloning techniques and verified by sequencing, is identical to the His-tagged Chimera with the exception of deleted residues 2-40. (**d, e, f**) Chimera and ChΔSc monomers were seeded by Chim[SC] and Chim[CA] fibers. (**g, h**) SC and CA monomers were seeded by ChΔSC[CA] fibers (compare to Chim[CA] in **Fig. 1b**).

Conclusions

Here we provide a structural explanation for earlier studies that established that the strain conformation of Chimera determines its seeding specificity for SC or CA Sup35. Specifically, we show that Chimera adopts two radically different conformations depending on the templating species. These two conformations have largely non-overlapping amyloid cores that are restricted to the species-specific region of Chimera, and are consistent with the locations of the short nucleating sequences identified by peptide array (Tessier and Lindquist 2007). Although both the SC- and CA-derived segments of Chimera are amyloidogenic, templating Chimera in one region appears to prevent amyloid formation in the other. Our observations provide a model for how the "molecular memory" of the species origin of the templating seed is recorded in the amyloid conformation of Chimera.

That a single polypeptide can form such radically different prion conformations substantially extends our view of the plasticity of protein misfolding. This together with related work showing structural diversity in amyloid formation (Sawaya *et al.* 2007; Toyama and Weissman 2011), emphasizes the inherent challenges of structural studies of prions and other amyloids, where subtle differences in polymerization conditions or the underlying peptide can result in dramatic changes in the resulting conformation. In addition, it also underscores the challenges in preventing the polymerization of such fibers. Given the multiplicity of nucleating sequences in amyloidogenic proteins, therapeutic strategies that selectively direct the energy landscape to favor less toxic amyloid conformations may be more successful than those that seek to abolish polymerization altogether.

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Author Contributions

C.K.F. and J.S.W. designed this study and wrote the manuscript. C.K.F. performed the hydrogen/deuterium exchange and truncation experiments. C.K.F. and M.J.S.K. performed the experiments for the NMR assignments. Y.O. and M.T. performed the limited proteolysis experiments.

Supplementary Information

Supplementary Methods

Assignment of Chimera spectrum. Sequence specific assignments of the backbone Hn and ^{15}N resonances were transferred from pre-existing assignments for corresponding SC residues (Toyama et al. 2007) where possible. Verification of transferred assignments and new assignments were obtained by using the following 3D triple resonance experiments on uniformly labeled ^{13}C-,^{15}N-Chimera: HNCO, HN(CA)CO, CBCA(CO)NH, HNCACB, HN(CA)NH, HNCA, HN(CO)CA. HNCO, HN(CA)CO, HNCA, and HN(CO)CA experiments used semi-constant time ^{15}N evolution and HNCA and HN(CO)CA additionally used constant time $^{13}C\alpha$ evolution to increase resolution (Yamazaki *et al.* 1994; Sun *et al.* 2005). All spectra were recorded on Bruker Avance 800 MHz or DRX 500 MHz spectrometers equipped with cryoprobes with actively shielded Z gradients at 298K. All NMR spectra were processed with nmrPipe (Delaglio *et al.* 1995) and assignments were performed using the program CcpNmr Analysis (Vranken *et al.* 2005).

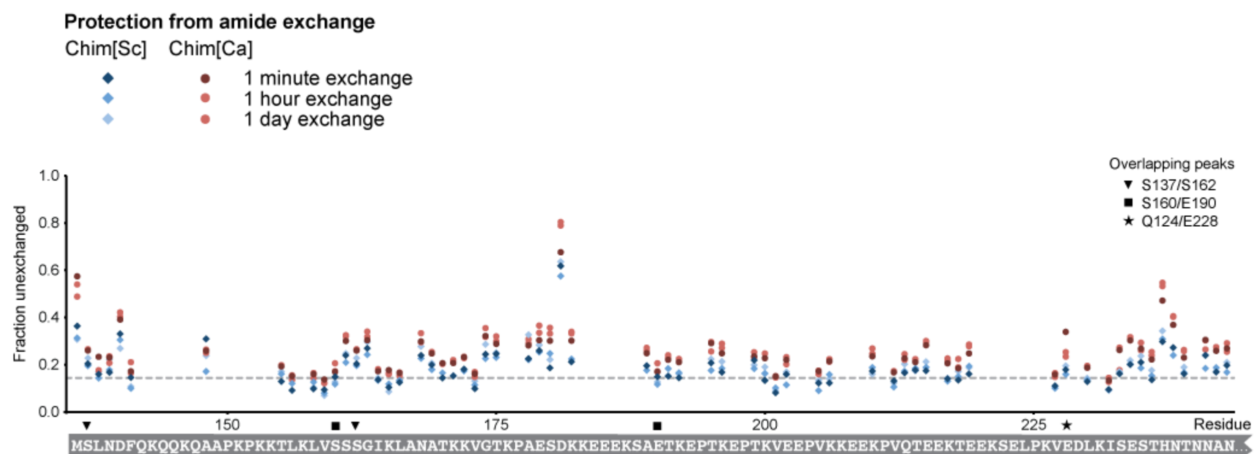

Figure S1. Protection from amide exchange for residues in the middle domain. Fractions unexchanged for Chimera residues 137-243 as described in the text.

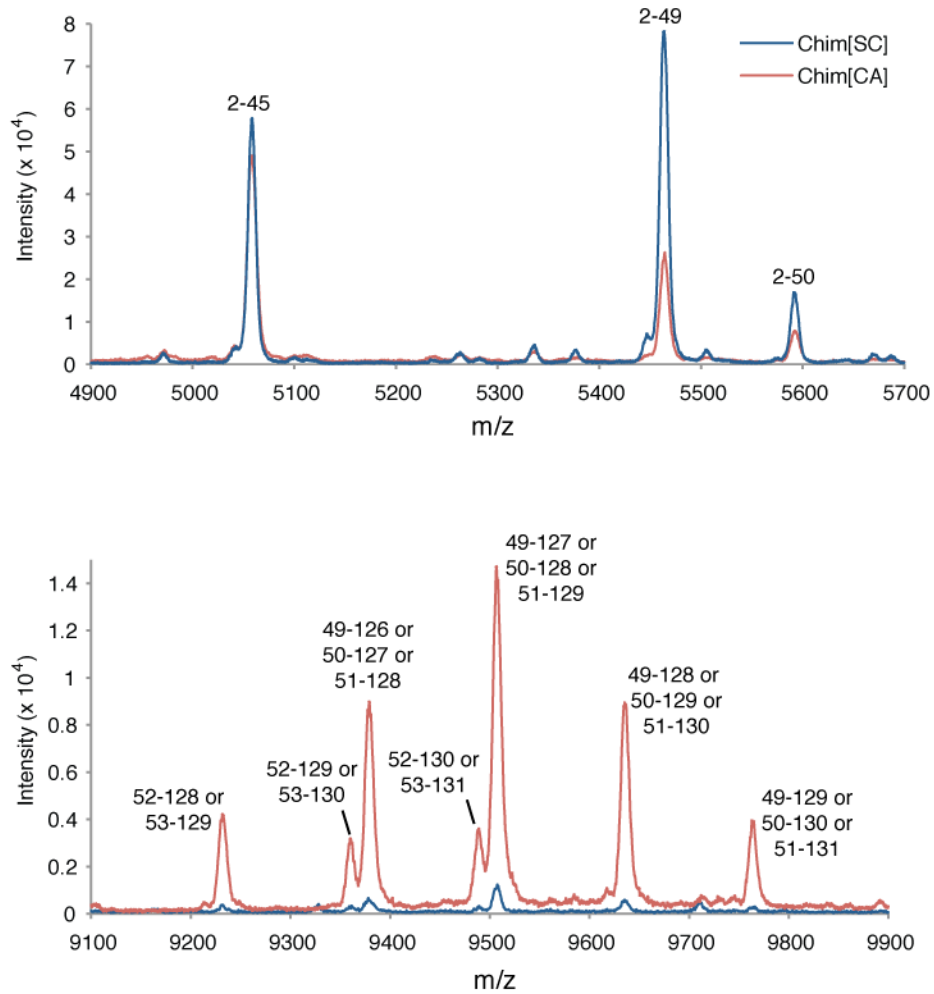

Figure S2. Limited proteolysis and MALDI-TOF MS. Shown here are the mass spectrometry data for proteolysis-resistant peptides from Chim[SC] and Chim[CA]. The peak for 2-50 is labeled here but due to the low intensity of the signal was not included in the schematic in Fig. 3b.

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Chapter IV

Uncovering the translational effects of [PSI^{+}] with ribosome profiling

Introduction

The [*PSI*$^+$] prion in *Saccharomyces cerevisiae* is due to the aggregation of Sup35 into a heritable aggregated form. Sup35 is a translation termination factor (eRF3), and aggregation of this protein in the [*PSI*$^+$] form reduces its activity, resulting in the readthrough of stop codons. Although *SUP35* is an essential gene and the [*PSI*$^+$] prion reduces its activity, the prion has no measurable effects on growth in lab strains grown under typical conditions (e.g. rich media such as YEPD)[1].

Comparing Sup35 sequences from different species indicates that the prion-forming domain of Sup35 is conserved across all budding yeasts (Kushnirov *et al.* 1990; Chernoff *et al.* 2000; Kushnirov *et al.* 2000; Santoso *et al.* 2000; Resende *et al.* 2002; Chen *et al.* 2007; Harrison *et al.* 2007). This suggests two hypotheses: either the prion-forming ability of Sup35 provides a selective advantage (Masel and Griswold 2009) or the ability of the domain to form prions is a by-product of another function of that domain (Edskes *et al.* 2009). In either case, if [*PSI*$^+$] is able to spontaneously appear in the wild, even at low frequency, then the ability to form [*PSI*$^+$] should have an impact on the evolution of the yeast genome.

Susan Lindquist and her laboratory have proposed that [*PSI*$^+$] is an "evolutionary capacitor" (Rutherford and Lindquist 1998; True and Lindquist 2000). In this model, mutations in non-coding sequences, including those just downstream of stop codons, accumulate due to the lack of selective pressures on those sequences. [*PSI*$^+$] acts as a switch to reveal the effects of these mutations by allowing translation of sequences after the stop

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[1] McGlinchey and colleagues (2011) argue that this is because current studies are biased against pathogenic variants of [*PSI*$^+$]. This suggests that there exists a threshold for Sup35 activity below which the cells are inviable, and that many variants of [*PSI*$^+$] can be stably propagated above this threshold.

codon. Under some stresses, the frequency of [PSI^+] induction appears to be elevated (Tyedmers *et al.* 2008). The model proposes that variation that is advantageous can increase in frequency in the population (and therefore become "fixed") and thus increase the fitness of the yeast. Since stop codons are highly evolutionarily labile (Giacomelli *et al.* 2007), these variations may become incorporated into coding sequences.

Regardless of the evolutionary consequences, the readthrough of stop codons may have significant effects on the cell (Von Der Haar and Tuite 2007). Although [PSI^+] has no discernable effect under standard laboratory conditions, [PSI^+]-dependent phenotypes can be identified under a variety of other conditions. In a pair of Nature papers (True and Lindquist 2000; True *et al.* 2004), Heather True catalogued a series of conditions that revealed [PSI^+]-dependent growth differences in a set of laboratory yeast strains. Although [PSI^+]-dependent effects were observable, very few conditions were consistent across all strains. For example, when grown on media containing lithium, [PSI^+] provided a growth advantage in the 5V-H19 strain when compared to the isogenic [psi^-] strain. However, [PSI^+] grew slower than isogenic [psi^-] strains in the 74D-694 strain, and in other strains, including D1142-1A, the cells grew at the same rate.

True found that these phenotypes were almost entirely attributable to the translation readthrough effects of [PSI^+] (True et al. 2004). When the endogenous copy of Sup35 was replaced with a partial loss-of-function allele ($Sup35^{C653R}$), many of the [PSI^+]-dependent phenotypes were observed in the [psi^-] cells. Correspondingly, Sup35 anti-suppressor (ASU) mutants such as $Sup35^{Q15R}$ also suppressed the [PSI^+]-dependent phenotypes in the [PSI^+] cells.

What is the genetic cause of these phenotypes? A few studies have attributed a number of these phenotypes to the effects of [PSI^+] on altering the translation of a few regulatory genes. For example, in strain 74D-694, [PSI^+] causes a frameshift that increases translation of Oaz1p, an antizyme that negatively regulates cellular polyamines. By replacing the frameshift context with that of another protein that is insensitive to [PSI^+], EST3, Namy and colleagues showed that 11 of 22 phenotypes observed could be attributed to the effect of [PSI^+] on Oaz1p (Namy *et al.* 2008) .

Another potential regulator of these phenotypes is Pde2p. Pde2p stability is compromised by the addition of 21 residues. Because Pde2p regulates cyclic AMP levels in the cell, removing Pde2p from the control of [PSI^+] has physiological effects including decreasing the cell's sensitivity to heat shock (Namy *et al.* 2002).

We sought to gain an understanding of how [PSI^+] regulates these phenotypes and decided to use a technique developed by our lab, ribosome profiling, which allows one to examine the location of translating ribosomes on a genome-wide scale (Ingolia *et al.* 2009). By examining the differences in ribosome occupancy in [psi^-] and [PSI^+] cells of different genetic backgrounds, we hope to elucidate the regulatory effects of [PSI^+].

Materials and methods

Yeast strains

Because we expected the effects of [PSI^+] to be highly dependent on the genetic background of the yeast, we wanted to use a set of genetically diverse yeast strains. We collaborated with Susan Lindquist and two members of her laboratory, Randal Halfmann and Dan Jarosz, who were investigating [PSI^+]-dependent phenotypes in genetically diverse yeast. In the following section I describe each of the yeast strains, their phenotypes, and the status of the genome sequencing. All yeast strains were obtained from R.H. and D.J. **Table 1** contains a summary with YCF indices.

All of the strains studied contain a fluorescent read-through reporter (see **Figure 1**). For each strain and [PSI^+] state, I selected 2-3 colonies that were separately transformed with the reporter. I confirmed the [PSI^+]-dependent GFP expression by flow cytometry in all strains (data not shown).

Laboratory strain W303

W303 (*can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*) is a laboratory yeast strain which facilitates genetic studies due to the ease of mating, sporulation, and mutagenesis via recombination. This strain contains the *ade2-1* mutation (GAA-to-TAA ochre mutation at codon 64), a color reporter for [PSI^+]. W303 was converted to [PSI^+] by the transformation of SupNM fibers formed at 4°C (Sc4). The [PSI^+] cells are sensitive to bleomycin (10 uM in YEPD at 23°C), SGalactose-CSM (2%), and SGlycerol-CSM (2%).

W303 has been sequenced by the Sanger Genome Resequencing Project[2]. Sequencing for this project was done at 1X to 3X coverage, with a focus on identifying single nucleotide polymorphisms (SNPs), and a provisional assembly exists.

Clinical isolate YJM653

YJM653 (MATa ΔHO::KanMX ΔURA3) is a clinical sample isolated from brocho-alveolar lavage. YJM653 [*PSI*$^+$] has improved growth in SGal-CSM (2%) when compared to isogenic [*psi*$^-$]. (D. Jarosz, personal communication.)

YJM653 is currently being sequenced by the Broad Institute and the sequences are being analyzed by Alex Lancaster in Susan Lindquist's laboratory.

Clinical isolate YJM326

Initially another clinical isolate, YJM326, was a candidate for investigation. However during passaging YJM326 was discovered to have lost the connection between its [*PSI*$^+$]-dependent phenotype and the presence of aggregated Sup35 (R. Halfmann, personal communication) and was thus excluded from further analysis.

Wild [PSI+] spore 17

UCD4D12 is a wine strain identified as [*PSI*$^+$] by Randal Halfmann, due to the presence of Sup35 aggregates by SDD-AGE (Halfmann and Lindquist 2008). This strain is

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[2] http://www.sanger.ac.uk/research/projects/genomeinformatics/sgrp.html Unfortunately the coverage for W303 is not very good, so the SNP calls are probably not very good either. In retrospect, we should have included this as a sequenced strain. It may be worth asking whether the Lindquist lab will be sequencing future samples, or consider doing this ourselves.

from U.C. Davis[3] with one copy of *URA3* knocked out with the hygromycin resistance cassette. Due to the complexity of working with polyploid cells in ribosome profiling, Halfmann sporulated the cells twice to obtain cells that appeared to contain near-haploid DNA content. Four spores were identified as strains of interest based on phenotypes (spores 3, 7, 17, and 51).

These spores are assumed to contain the pre-existing [PSI^+] strain, which appears to be a strong prion strain when assessing read-through using a fluorescent reporter. Note that the presence of [PSI^+] has not been directly tested by SDD-AGE as of November 2010 (R. Halfmann, personal communication). The isogenic [psi^-] strains were created by curing [PSI^+] by growing the cells in the presence a low concentration of guanidine hydrochloride. If these cells contain other pre-existing prions, it is possible that the other prions may also be disrupted by this treatment, and the results must be interpreted with care to avoid conflating the effects of different prions. We can, for example, confirm that the phenotypes are dependent on readthrough by introducing an anti-suppressing (ASU) Sup35 mutant into the [PSI^+] background (also see techniques used in True *et al.* 2004).

Spore 17 has [PSI^+]-dependent fluconazole resistance and rapamycin resistance. (D. Jarosz, personal communication.) In addition, it also has a [PSI^+]-dependent invasive growth phenotype. This spore was initially selected for further investigation due to these phenotypes, and we plan to follow up with parallel investigations of the other strains at a later date.

Spores 3, 7, and 17 have been sequenced by the Broad Institute and the sequences are being analyzed by Alex Lancaster in Susan Lindquist's laboratory.

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[3] UC Davis Department of Viticulture & Enology. Website: http://wineserver.ucdavis.edu/content.php?category=Research&id=367

Genetic diversity of yeast strains

Preliminary analysis of the sequencing data for YJM653 and spore 17 have revealed that each have about 10,000 nonsynonomous coding changes, suggesting that these strains are approximately genomically equidistant from each other. (D. Jarosz, personal communication)

Ribosome profiling

Ribosome profiling was performed as described previously (Ingolia et al. 2009) with modifications developed by Gloria Brar and Silvia Rouskin. Yeast cells were inoculated from a plate into a small liquid culture of YEPD + hygromycin, for selection of the fluorescent reporter plasmid. The cells were then inoculated into 750ml YEPD at an OD600 of 0.15 and allowed to grow until an OD600 of 0.6. Cells were treated with cyclohexamide, frozen in liquid nitrogen and lysed by mixermilling. Unprotected RNA is digested with RNaseI, leaving only the protected RNA, including the mRNA fragments protected by ribosomes (Steitz 1969). Assembled 80S ribosomes and their protected mRNA fragments are isolated with a sucrose gradient, and the RNA is subsequently extracted. In a deviation from the original protocol, a linker is ligated to the 3' end of the isolated RNA fragments, rather than the polyadenylation step described previously. (Primers used in further steps downstream are also altered to accommodate the linker sequence.) Ribosomal RNA sequences can co-purify with the mRNA footprints in very high quantities, up to 90% (Ingolia et al. 2009), and therefore, in another alteration to the original protocol, very high frequency sequences are removed using a cocktail of a few complementary oligonucleotides bound to magnetic beads. The remaining sequences are reverse transcribed to cDNA, the

RNA fragments are hydrolyzed, and the remaining cDNA is circularized. These DNA circles are now PCR amplified and the result is a strand-specific library, compatible with Illumina next-generation sequencers, representing the ribosome footprints.

For each strain, at least two biological duplicates were prepared and sequenced on the Illumina GenomeAnalyzerII (GAII) and/or the Illumina HiSeq using primer ONTI202. Sequencing data available is described in **Table 2**.

Analysis of sequencing data

This section describes the processing of the reads generated by the CASAVA pipeline into alignments. Because the protocol that we use ligates a linker sequence to the 3' end of every RNA fragment, we can unambiguously identify the 3' end by locating the linker sequence within each read. First, all positions that map to the initial 7 nucleotides of the linker sequence, allowing for up to one mismatch, are located within the read. The rest of the linker sequence is then matched to the read, allowing a 20% mismatch.

Reads obtained from sequencing on the GAII were typically 40bp whereas the reads from the HiSeq are 50bp, allowing for better resolution of the linker location. Reads where a linker could not be identified, or those that would result in a very short read (under 18bp) were removed from further analysis.

Next, the reads were processed to remove sequences that aligned to oligonucleotides used in library generation ("markers") and ribosome RNA sequences from the reference yeast genome. For these and all other alignments, the first two nucleotides were trimmed before aligning. Empirically I observed an elevated rate of mismatch for the first two nucleotides, possibly due to the stochastic addition of an untemplated base by reverse

transcriptase (data not shown). The sequences were aligned with the Bowtie aligner (Langmead *et al.* 2009). Up to two mismatches were permitted[4].

The remaining reads were aligned to the reference sequence in successive rounds, allowing for increasing mismatches (using the end-to-end mismatching with the –v parameter[5]). Only unique alignments were further analyzed.

Mapping reads

Ideally we would like to be able to map each footprint to the codon that it is actively translating, even to determine the frame. In Ingolia *et al.*, (2009) a fixed offset from the 5' end was used because the polyadenylation prevented many transcripts from having a resolved 3' end. However, the linker ligation step should allow us to determine the 3' end. This also avoids confusion due to the frequent addition of untemplated nucleotides at the 5' end, observed by me (as mentioned above) and reported by many members of the Weissman lab (personal communication from C. Jan, G. Li, B. Weisburd).

Calculating ribosome density

Ribosome density is calculated in terms of reads per kilobase of sequence per million alignable reads (rpkM). When only mapping uniquely alignable reads, the sequence length should be corrected to only account for unique sequence.

[4] bowtie arguments: --phred64-quals -a -q -5 2 -p 2 -v 2
[5] bowtie arguments: --phred64-quals -m 1 -q -5 2 -p 2 -v {0,1,2,3}

Figure 1: pAG41 GPD GST-stop-EGFP

This plasmid was created by Randal Halfmann in the Lindquist laboratory. This plasmid is in my database as pCF8. An identical plasmid bearing the NAT cassette for nourseothricin resistance is named pCF9. A version of this plasmid with the URA marker also exists.

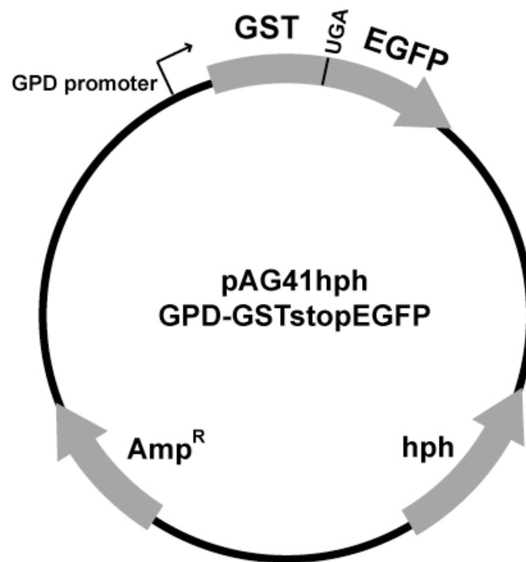

Table 1: Yeast strains by YCF index

Yeast strain background	*PSI* state	*PSI* strain	Without GFP[6]	With GFP
W303	*psi- pin-*	none	YCF173	YCF181
			YCF174	YCF182
	PSI+ pin-	Sc4[7]	YCF161	YCF183
			YCF162	YCF184
YJM653	*psi- pin-*	none	YCF167	YCF185
			YCF168	YCF186
	PSI+ pin-	Sc4	YCF165	YCF187
			YCF166	YCF188
Spore 17	*psi- pin-*	none	YCF204	YCF216
			YCF205	YCF217
			YCF206	YCF218
	PSI+ pin?	endogenous[8]	YCF201	YCF213
			YCF202	YCF214
			YCF203	YCF215
Spore 3	*psi- pin-*	none	YCF192	*NA*[9]
			YCF193	*NA*
			YCF194	*NA*
	PSI+ pin?	endogenous	YCF189	*NA*
			YCF190	*NA*
			YCF191	*NA*
Spore 7	*psi- pin-*	none	YCF198	*NA*
			YCF199	*NA*
			YCF200	*NA*
	PSI+ pin?	endogenous	YCF195	*NA*
			YCF196	*NA*
			YCF197	*NA*
Spore 51	*psi- pin-*	none	YCF210	YCF223/224[10]
			YCF212	YCF225/226
	PSI+	endogenous	YCF208	YCF219/220
			YCF209	YCF221/222

[6] *Without GFP* represents strains frozen down from cultures that the Lindquist lab sent me. *With GFP* represents the resulting strain after transforming the GFP reporter plasmid into the corresponding strain from *Without GFP*.
[7] Transformed by the Lindquist lab.
[8] *endogenous* indicates that this [*PSI*$^+$] strain was originally in the yeast strain. As a result, this yeast strain may contain other prions and the [*PIN*$^+$] state is unknown.
[9] *NA* indicates that this yeast strain has not been created yet.
[10] For some reason I felt compelled to keep two isolates of these transformations.

Table 2: Sequencing data currently available

Sample	Date/Seq[11]	Lane[12]	Total reads	Aligned footprints[13]	Notes[14]
YCF181 FP	100426 G	7 (Nick)	19.7 M	14.4 M	13.3.16.1
	110408 H	1 (Martin)	81.9 M	60.0 M	
YCF182 FP	100517 G	1 (Andrew)	20.5 M[15]	9.6 M	see [17]
	110222 H	5	87.0 M[16]	48.5 M	
YCF183 FP	100426 G	6 (Nick)	20.2 M	12.1 M	13.3.16.3
	110408 H	2 (Martin)	83.5 M	49.6 M	
YCF184 FP	100517 G	2 (Andrew)	20.9 M[15]	9.1 M	see [17]
	110222 H	6	87.2 M[16]	48.7 M	
YCF185 FP	100517 G	3 (Andrew)	20.0 M	13.4 M	
YCF186 FP	100809 G	7 (Nick)	22.7 M	17.4 M	
	110222 H	7	32.8 M	--	see [18]
YCF186 FP	110331 H	5 (Calvin)	93.8 M	69.1 M	see [19]
YCF187 FP	100517 G	4 (Andrew)	18.9 M	11.8 M	
YCF188 FP	110131 G	7 (Eugene)	21.5 M	14.0 M	13.4.16.4
	110222 H	8	--	--	see [18]
YCF188 FP	110331 H	6 (Calvin)	94.1 M	61.8 M	see [20]
Spore17 A FP	110504 H	6	23.6 M	12.0 M	CAGATC
Spore17 B FP	110504 H	6	22.8 M	11.9 M	GCCAAT
YCF216 FP	110504 H	6	14.4 M	7.2 M	ATCACG
YCF217 FP	110504 H	6	17.9 M	9.5 M	TGACCA

Note: YJM653 samples were sequenced on the GAII on 100201 (YCF146/147); however, these samples turned out to be both [*PSI*$^+$] by western blot (see my lab notebook 5 page 83).

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[11] YYMMDD. G, sequenced on Illumina GenomeAnalyzer II; H, Illumina HiSeq

[12] Name in parentheses indicates the person in charge of the flow cell, when recorded. Nicolas Ingolia, Martin Kampmann, Eugene Oh, Dale Muzzey, Andrew Hsieh

[13] Aligned to S288C reference genome as described in Methods. Some counts are from older alignments using Nick Ingolia's tagalign.

[14] 13.X are sample numbers. For multiplexed samples, barcode is recorded in Notes column.

[15] Read counts are for entire lane including spiked-in sample.

[16] Some read counts are after polyA and marker filtering; difference is minimal.

[17] This lane has 20% of a polio sample from Silvia Rouskin.

[18] Two lanes were affected by a fluidics problem and were re-run at a later date.

[19] Identical sample multiplexed four ways as a control by Calvin Jan. Amplified from circles.

[20] Same as 19, but amplified from library.

Table 2, continued

Sample	**Date/Seq**	**Lane**	**Total reads**	**Aligned footprints**	**Notes**
YCF181 mRNA	110504 H	5	10.1 M	2.8 M	ATCACG
YCF182 mRNA	101115 G	6 (Dale)	14.4 M	4.2 M	see [21]
YCF182 mRNA	110504 H	5	9.3 M	2.9 M	TGACCA
YCF183 mRNA	110504 H	5	10.7 M	3.5 M	CAGATC
YCF184 mRNA	101115 G	7 (Dale)	9.9 M	3.2 M	see [21]
YCF184 mRNA	110504 H	5	12.2 M	4.8 M	GCCAAT
YCF185 mRNA	110131 G	5 (Eugene)	6.7 M	1.9 M	big clusters
YCF185 mRNA	110504 H	5	11.0 M	3.5 M	ACTTGA
YCF186 mRNA	110131 G	6 (Eugene)	19.9 M	6.3 M	
YCF186 mRNA	110504 H	5	7.6 M	2.8 M	TAGCTT
YCF187 mRNA	110504 H	5	8.4 M[22]	2.9 M	GGCTAC
YCF188 mRNA	110504 H	5	9.5 M	2.9 M	CTTGTA

Note: no mRNA samples for spore17 have been prepared.

[21] Two lanes were affected by an undocumented problem; samples were re-prepped by E. Oh and run as a multiplexed lane at a later date.
[22] Includes 1.0 M reads that have barcode GGCTAA (one error); those reads are not included in aligned counts.

Preliminary results

Repeatability of data

Figure 2 shows normalized ribosome density for W303 [*PSI*$^+$] and [*psi*$^-$] samples. The correlation of for all the graphs is very good. Qualitatively more data points appear to deviate from the $y = x$ line in the [*PSI*$^+$] vs [*psi*$^-$] graph (**Figure 2c**) and those are candidates for further exploration.

[*PSI*$^+$] controls

To confirm the [*PSI*$^+$] state, reads were also aligned to the GST-stop-EGFP reporter plasmid that each strain contained. Readthrough was calculated as the reads per kilobase per million alignable reads (rpkM) ratio of GST compared to EGFP. [*psi*$^-$] samples observed to have 1% readthrough, possibly caused by reinitation due to the internal ATG at the start of EGFP. The presence of [*PSI*$^+$] induces an increase to about 5% readthrough. This level of increase in readthrough is consistent with previous observations with different reporters (Firoozan *et al.* 1991; Namy et al. 2002; Namy *et al.* 2003; Namy et al. 2008).

We expected that *ade2-1* would serve as a control of [*PSI*$^+$] status in W303, but no readthrough was observed. There are at least two possible explanations: rich media may not induce the adenine biosynthesis pathway, or growth under stress conditions amplify the effect of [*PSI*$^+$] either by increasing the basal level of translation or specifically enhancing the translation termination defect of [*PSI*$^+$].

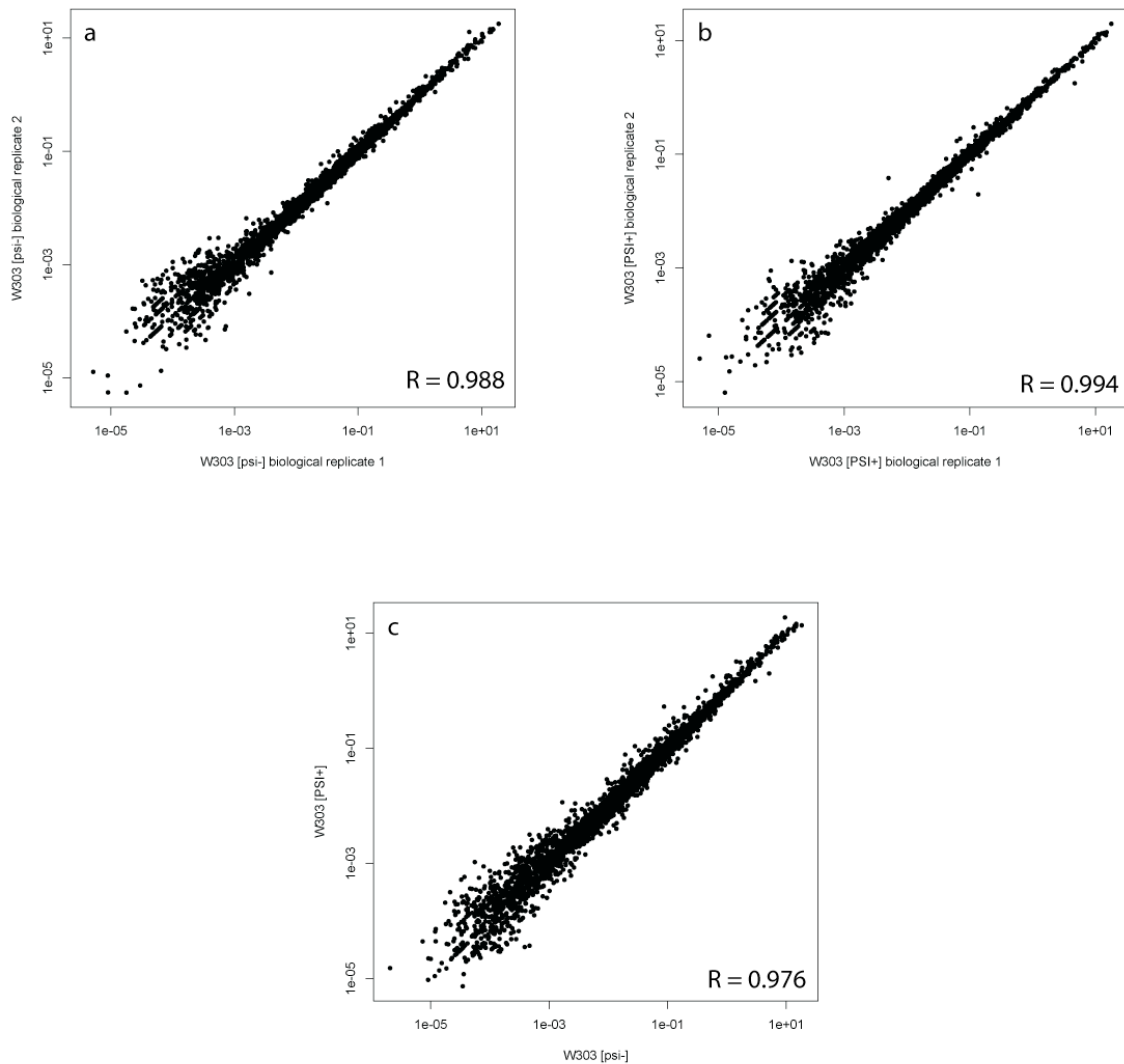
Visual inspection of *PDE2* and *OAZ1* suggests [PSI+]-dependent readthrough and frameshift, respectively, but more quantitative measures are necessary.

Figure 2: W303 gene expression.

(a) Two biological replicates of W303 [*psi*], YCF181 and YCF182, were ribosome profiled. For each annotated gene, reads per kilobase per million aligned reads is plotted. R is pearson correlation.

(b) YCF183 and YCF184.

(c) YCF181/182 vs YCF183/184.



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Chapter V

Conclusions

In the less than two decades since Reed Wickner proposed that [URE3] and [PSI^+] were prions in yeast (Wickner 1994), we have uncovered many of the molecular mechanisms that govern prion propagation. We now understand that prions are infectious amyloid fibers that can be divided by the cellular machinery that typically acts to prevent the accumulation of harmful aggregates (True 2006). We know that prion strains can be encoded within the conformation of these fibers (Tanaka *et al.* 2004; Collinge and Clarke 2007; Toyama *et al.* 2007; Tessier and Lindquist 2009). We have demonstrated that infectious prions can be generated *in vitro* using pure recombinant protein (Tanaka et al. 2004). All of these discoveries have increased our understanding of mammalian prions.

[PSI^+] has provided a convenient test bed for evaluating the prion transmission barrier between different protein sequences, allowing us to uncover the molecular basis of this barrier (Chien *et al.* 2004). One particular fusion of the *Saccharomyces cerevisiae* Sup35 protein and its homolog in *Candida albicans* has been particularly revealing (Santoso *et al.* 2000; Chien and Weissman 2001; Chien *et al.* 2003; Tessier and Lindquist 2007). Initially, this chimeric protein's ability to cross the species barrier was puzzling; by using hydrogen/deuterium exchange and nuclear magnetic resonance (Hoshino *et al.* 2002; Toyama et al. 2007) we discovered that this is due to its ability to form amyloid cores in mutually exclusive regions (Foo *et al.* 2011).

In addition, we now know that the yeast cell can contain numerous other prions (Du *et al.* 2008; Alberti *et al.* 2009; Sindi and Serio 2009). Many of the proteins that have prion-forming domains are transcription factors or RNA-binding proteins, suggesting that prions may act as regulatory switches for these pathways (Du *et al.* 2008; Alberti *et al.* 2009). This may indicate that prions play a larger role in the regulation of cellular processes than

previously appreciated. We hope to soon have a greater understanding of what regulatory roles [*PSI*$^+$] may play in the yeast *S. cerevisiae* (Chapter 4).

In any case, the existence of so many sequences in the yeast genome that can form prions suggests that prion formation plays a critical and yet unidentified role. Alternatively, we can hypothesize that the prion-forming ability is a side effect of some other protein-protein interaction mediated by these sequences (Bailleul *et al.* 1999; Cosson *et al.* 2002; Urakov *et al.* 2006; Volkov *et al.* 2007). Even so, these prions still influence the evolution of yeast (Griswold and Masel 2009; Masel and Griswold 2009), as the cells must have some mechanism to prevent undesired aggregation of these peptides.

I have not discussed the other well-studied prion of another yeast species, the [Het-S] prion in *P. anserina*. Unlike Sup35, HET-s does not contain an asparagine/glutamine-rich sequence in its prion domain. Therefore, the biophysical properties that govern its behavior are very different. In addition, the function of [Het-S] is clear: it allows for heterokaryon identification in mating. The question remains open: are there other prions in yeast including *Saccharomyces cerevisiae* that look more like [Het-S] and less like [*PSI*$^+$]? In one interesting set of experiments, the oligopeptide repeat of Sup35 can be functionally replaced by a non-Asn/Gln rich sequence; however, this alteration causes the resulting prion to become HSP104 independent (Crist *et al.* 2003).

This question can also be extended to humans and other mammals. We know that PrP, in its prion form, can cause a variety of transmissible spongiform encephalopathies. Might there also be functional prions playing a role in some mammalian cells? Although prion diseases have been studied for centuries (Schwartz 2003), it is only within the past generation that we have begun to understand their puzzling nature. It is possible that prions

could represent a generalizable aspect of cellular regulation that we are only beginning to understand.

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Appendix A

Development of a fluorescence-tagged Sup35

Introduction

Fluorescence labeling is a common and effective means of investigating the localization of various proteins. A green fluorescent protein (GFP)-tagged Sup35 allows the visualization of Sup35 aggregates in vivo, allowing for the investigation of the form, number, localization, and changes in [*PSI*$^+$] prion aggregates.

In this section, I will review the domain structure of Sup35 and other GFP-tagged Sup35 constructs described in the literature. I will then explain the rationale behind the design of this particular construct and explain its construction and validation. Based on conservation and the crystal structure of the Sup35 eRF3 domain from *Schizosaccharomyces pombe*, we chose to insert the tag after residue E216. I created and tested a series of plasmids with restriction sites for the insertion of any tag at this location that were designed for integration into the yeast genome.

Sup35 domain structure

The Sup35 protein has classically been divided into three domains based on the presence of two internal methionines (Kushnirov *et al.* 1988). The N domain is considered the prion-forming domain, the M domain is the "charged" middle domain, and the C domain contains the translation termination activity.

Existing Sup35-GFP fusions

Two Sup35-GFP constructs exist in the literature. One, NM-GFP, consists of the N and M domains of Sup35 fused to GFP (Patino *et al.* 1996). This construct does not contain the C domain and therefore cannot replace endogenous Sup35. This construct has been

used to identify and characterize "decorated" [*PSI*$^+$] aggregates (Patino et al. 1996; Glover *et al.* 1997). The second, Sup35-GFP, contains a GFP located between the N and M domains of a full length Sup35 protein (Satpute-Krishnan and Serio 2005). Sup35-GFP can be integrated into the genomic locus and has been used to monitor prion conversion in real time (Satpute-Krishnan and Serio 2005; Satpute-Krishnan *et al.* 2007; Pezza *et al.* 2009; Derdowski *et al.* 2010; Disalvo *et al.* 2011).

Selection of site for Sup35 fluorescent tagging

Our laboratory's recent work characterizing Sup35 amyloids by hydrogen-deuterium exchange and NMR revealed that the residues critical for amyloid structure could vary among the studied conformations. To avoid any potential conformation-specific effects, we decided to create a new Sup35-GFP fusion with the GFP tag integrated farther from the N domain.

Although the structure for *S. cerevisiae* Sup35 (ScSup35) is not known, the crystal structure of the eRF3 domain of the Sup35 homolog in *Schizosaccharomyces pombe* (SpSup35) has been solved (Kong *et al.* 2004). A truncation of SpSup35 was used that began at residue S196 (corresponding residue in ScSup35 is around L212, although conservation in this region is low and therefore not reliable). Structure could be observed beginning at residue T215 (corresponding to ScSup35 D236). Therefore we decided that an appropriate junction for tag insertion would be upstream of this location.

In addition, Kim Tipton performed a Clustalw (Chenna *et al.* 2003) alignment on Sup35 from several species (a similar alignment is reproduced as **Figure 1**; *S. cerevisiae, Ashbya*

gossypii, Kluyveromyces lactis, Candida glabrata, Candida albicans, Yarrowia lipolytica, S. pombe). We observed a high degree of conservation for residues downstream of ScSup35 D236.

With the crystal structure and conversation of the EF domain of Sup35, we chose to insert a site adjacent to ScSup35 E216.

Genomic integration of fluorescent-tagged Sup35

To study the behavior of Sup35 under endogenous levels, we wanted to integrate the tagged *SUP35* into the genome under its endogenous promoter. Unfortunately, attempts at genomic integration were unsuccessful.

Conclusions

Fluorescence-tagged Sup35 is a useful tool for investigating the behavior of Sup35 aggregates in [PSI^+] cells. In this chapter, I have described efforts to create a tagged Sup35 that is located far from the prion-forming N domain and upstream of any conservation or predicted structure in the eRF3 domain. Although the tagged construct has not yet been integrated into the genome, these plasmids have already been used by others in the laboratory as a functional C-terminal Sup35 truncation.

In addition to providing a tagged construct for use in fluorescence studies, I also created a truncation that should be identical in translation termination activity as the full-length Sup35p, and therefore could be used in an EMAP study (Schuldiner *et al.* 2005) to understand the non-prion related function of the N domain (Bailleul *et al.* 1999; Cosson *et al.* 2002; Urakov *et al.* 2006; Volkov *et al.* 2007).

Figure 1. Clustalw alignment of Sup35p, page 1 of 3

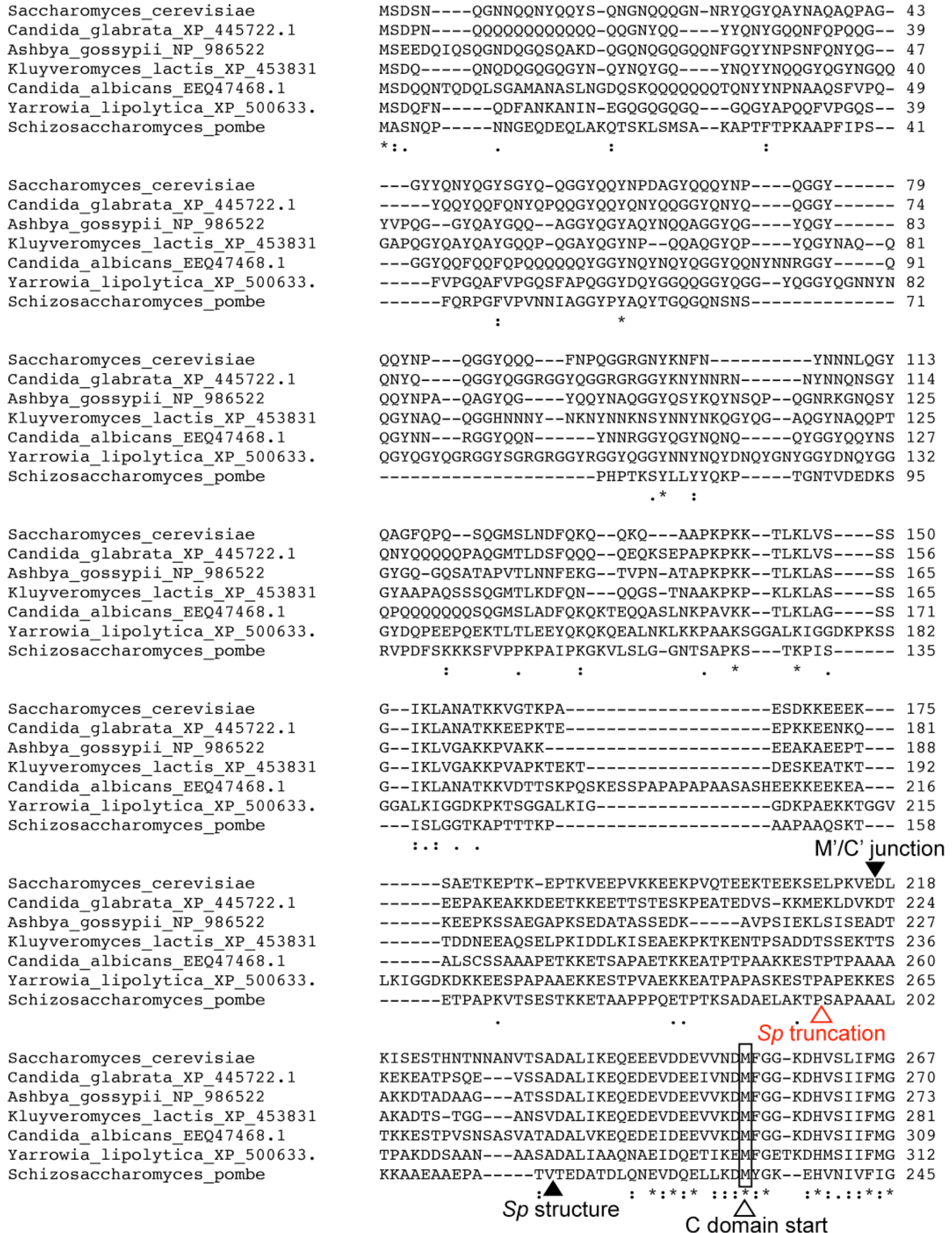

Figure 1. Clustalw alignment of Sup35p, page 2 of 3

```
Saccharomyces_cerevisiae          HVDAGKSTMGGNLLYLTGSVDKRTIEKYEREAKDAGRQGWYLSWVMDTNK 317
Candida_glabrata_XP_445722.1      HVDAGKSTMGGNLLYLTGSVDKRTIEKYEREAKDAGRQGWYLSWVMDTNK 320
Ashbya_gossypii_NP_986522         HVDAGKSTMGGNLLYLTGSVDKRTVEKYEKEAKEAGRQGWYLSWIMDTNK 323
Kluyveromyces_lactis_XP_453831    HVDAGKSTMGGNLLYLTGSVDKRTVEKYEREAKEAGRQGWYLSWVMDTNK 331
Candida_albicans_EEQ47468.1       HVDAGKSTMGGNILYLTGSVDKRTVEKYEREAKDAGRQGWYLSWVMDTNK 359
Yarrowia_lipolytica_XP_500633.    HVDVGKSTLGGQILYLTGTVNKRTIEKMEKEAADAGRPGWYLSWVMDINK 362
Schizosaccharomyces_pombe         HVDAGKSTLGGNILFLTGMVDKRTMEKIEREAKEAGKESWYLSWALDSTS 295
                                  ***.****:**::*:*** *:***:** *:** :**: .***** :* ..

Saccharomyces_cerevisiae          EERNDGKTIEVGKAYFETEKRRYTILDAPGHKMYVSEMIGGASQADVGVL 367
Candida_glabrata_XP_445722.1      EERNDGKTIEVGKAYFETEKRRYTILDAPGHKMYVSEMIGGASQADVGIL 370
Ashbya_gossypii_NP_986522         EERNDGKTIEVGRSYFETEKRRYTILDAPGHKMYVSEMIGGASQADVGIL 373
Kluyveromyces_lactis_XP_453831    EERNDGKTIEVGRAYFETEKRRYTILDAPGHKMYVSEMIGGASQADIGIL 381
Candida_albicans_EEQ47468.1       EERNDGKTIEVGKAYFETDKRRYTILDAPGHKMYVSEMIGGASQADVGIL 409
Yarrowia_lipolytica_XP_500633.    EERAEGKTVEVGRSYFETDKRRYTLLDAPGHKMYVPSMIGGAAQADAGIL 412
Schizosaccharomyces_pombe         EEREKGKTVEVGRAYFETEHRRFSLLDAPGHKGYVTNMINGASQADIGVL 345
                                  *** .***:***::****::**:::******* **..**.**:*** *:*

Saccharomyces_cerevisiae          VISARKGEYETGFERGGQTREHALLAKTQGVNKMVVVVNKMDDPTVNWSK 417
Candida_glabrata_XP_445722.1      VISARKGEYETGFEKGGQTREHALLAKTQGVNKMIVVINKMDDPTVNWSQ 420
Ashbya_gossypii_NP_986522         VISARKGEYETGFERGGQTREHALLAKTQGVNKMVVVVNKMDDPTVNWDK 423
Kluyveromyces_lactis_XP_453831    VISARKGEYETGFEKGGQTREHALLAKTQGVNKMIVVINKMDDPTVGWDK 431
Candida_albicans_EEQ47468.1       VISARKGEYETGFEKGGQTREHALLAKTQGVNKIIVVVNKMDDSTVGWSK 459
Yarrowia_lipolytica_XP_500633.    VISARKGEYETGFERDGQTREHAMLAKTQGINKLVIAINKMDDSTVNWSK 462
Schizosaccharomyces_pombe         VISARRGEFEAGFERGGQTREHAVLARTQGINHLVVVINKMDEPSVQWSE 395
                                  *****:**:*:***:.*******:**:***:*::::.:****:.:* *.:

Saccharomyces_cerevisiae          ERYDQCVSNVSNFLR-AIGYNIKTDVVFMPVSGYSGANLKDHVDPKECPW 466
Candida_glabrata_XP_445722.1      ERYDQCVSNLSNYLK-AIGYNVKQDVVFMPVSGYSGAGLKERVKKEECPW 469
Ashbya_gossypii_NP_986522         ARYDQCIKNVSNFLQ-AIGYNVKEDVMYMPVSGFTGAGLKDRVDKKDCPW 472
Kluyveromyces_lactis_XP_453831    ERYDHCVGNLTNFLK-AVGYNVKEDVIFMPVSGYTGAGLKERVDPKDCPW 480
Candida_albicans_EEQ47468.1       ERYQECTTKLGAFLK-GIGY-AKDDIIYMPVSGYTGAGLKDRVDPKDCPW 507
Yarrowia_lipolytica_XP_500633.    ERYDECISKLKLYLK-GLGYSDKTEITFMPVSGFTGANVKERVSKEVCPW 511
Schizosaccharomyces_pombe         ERYKECVDKLSMFLRRVAGYNSKTDVKYMPVSAYTGQNVKDRVDSSVCPW 445
                                   **..*  ::  :*:   **  * :: :****.::* .:*::*. . ***

Saccharomyces_cerevisiae          YTGPTLLEYLDTMNHVDRHINAPFMLPIAAKMKDLGTIVEGKIESGHIKK 516
Candida_glabrata_XP_445722.1      YDGPALLEYLDEMKHVDRHVNAPFMLPIASKMKDLGTVVEGKIESGHIKK 519
Ashbya_gossypii_NP_986522         YDGPSLLEYLDNMQHVDRFINAPFMLPIASKMKDMGTVVEGKIESGHIKK 522
Kluyveromyces_lactis_XP_453831    YTGPSLLEYLDNMKTTDRHINAPFMLPIASKMKDMGTVVEGKIESGHIRK 530
Candida_albicans_EEQ47468.1       YDGPSLLEYLDNMDTMNRKINGPFMMPVSGKMKDLGTIVEGKIESGHVKK 557
Yarrowia_lipolytica_XP_500633.    YDGPSLLELLDSFE-LERNLTGPFMLPISNKEKNLGTIVEGKIEVGTVKK 560
Schizosaccharomyces_pombe         YQGPSLLEYLDSMTHLERKVNAPFIMPIASKYKDLGTILEGKIEAGSIKK 495
                                  * **:*** ** :   :* :..**::*:: * *::**::***** * ::*

Saccharomyces_cerevisiae          GQSTLLMPNKTAVEIQNIYNETENEVDMAMCGEQVKLRIKGVEEEDISPG 566
Candida_glabrata_XP_445722.1      GQSTLLMPNKIPVEIQNIYNETENEVDMAVCGEQVKLRIKGIEEEDISAG 569
Ashbya_gossypii_NP_986522         GNQTLLMPNKIPVEILAIQNETEQEVDMAVCGEQVRLRLKGVEEEDISAG 572
Kluyveromyces_lactis_XP_453831    GNQTLLMPNRTSVEILTIYNETESEVDMAVCGEQVRLRIKGVEEEEISAG 580
Candida_albicans_EEQ47468.1       GTNLIMMPNKTPIEVLTIFNETEQECDTAFSGEQVRLKIKGIEEEDLQPG 607
Yarrowia_lipolytica_XP_500633.    GDNLVVMPSKVPVEVTTLYKETEQEVGGASVGEQIRLKVKGIEEEEVQIG 610
Schizosaccharomyces_pombe         NSNVLVMPINQTLEVTAIYDEADEEISSSICGDQVRLRVRG-DDSDVQTG 544
                                  . . ::** . .:*:  : .*::.* . :  *:*::*:::* ::.::. *

Saccharomyces_cerevisiae          FVLTSPKNPIKSVTKFVAQIAIVELKSIIAAGFSCVMHVHTAIEEVHIVK 616
Candida_glabrata_XP_445722.1      FVLTSPKNPIKNVTRFVAQIAIVELKSIMSAGFSCVMHVHTAIEEVHITR 619
Ashbya_gossypii_NP_986522         FVLTSPKNPVKNVTKFVAQIAIVELKSIMSAGFSCVMHVHTAIEEVSITR 622
Kluyveromyces_lactis_XP_453831    FVLTSPKNPVKNVTRFVAQIAIVELKSIMSAGFSCVMHIHTAIEEVTVTR 630
Candida_albicans_EEQ47468.1       YVLTSPKNPVKTVTRFEAQIAIVELKSILSNGFSCVMHLHTAIEEVKFIE 657
Yarrowia_lipolytica_XP_500633.    QVLCSAAQPVAAVTVFEAQIAITELKSILSTGFSCVMHIHTAAEEVTFTA 660
Schizosaccharomyces_pombe         YVLTSTKNPVHATTRFIAQIAILELPSILTTGYSCVMHIHTAVEGVSFAK 594
                                   ** *. :*:  .* * ***** ** **:: *:*****:*** * * .  
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Figure 1. Clustalw alignment of Sup35p, page 3 of 3

```
Saccharomyces_cerevisiae            LLHKLEKGTNRKSKKPPAFAKKGMKVIAVLETEAPVCVETYQDYPQLGRF 666
Candida_glabrata_XP_445722.1        LLHKLERGTNRKSKKPPAFAKKGMKIIALLETEAPVCVETYDDYPQLGRF 669
Ashbya_gossypii_NP_986522           LLHKLEKGTNRKSKKPPAFAKKGMKIIAVLETEEPVCVETYQDYPHLGRF 672
Kluyveromyces_lactis_XP_453831      LLHKLEKGSNRKSKKPPAFAKKGMKIIAVIETNEPVCVETYDDYPQLGRF 680
Candida_albicans_EEQ47468.1         LKHKLEKGTNRKSKKPPAFAKKGMKIIAILEVGELVCAETYKDYPQLGRF 707
Yarrowia_lipolytica_XP_500633.      LLHKLEKGTNRKSKKPPAFAKKGMKIIARLETQNPVCMDEFSKTPQLGRF 710
Schizosaccharomyces_pombe           LLHKLDK-TNRKSKKPPMFATKGMKIIAELETQTPVCMERFEDYQYMGRF 643
                                    * ***:: :******** **.****:** :*.   ** : :..   :***

Saccharomyces_cerevisiae            TLRDQGTTIAIGKIVKIAE- 685
Candida_glabrata_XP_445722.1        TLRDQGTTIAIGKIVKILE- 688
Ashbya_gossypii_NP_986522           TLRDQGITIAIGKIVKILE- 691
Kluyveromyces_lactis_XP_453831      TLRDQGTTIAIGKIVKILEN 700
Candida_albicans_EEQ47468.1         TLRDQGTTIAIGKITKLL-- 725
Yarrowia_lipolytica_XP_500633.      TLRDQGQSIAIGRVTKLL-- 728
Schizosaccharomyces_pombe           TLRDQGTTVAVGKVVKILD- 662
                                    ****** ::*:*::.*:
```

Sup35p sequences from *Saccharomyces cerevisiae*, *Ashbya gossypii*, *Kluyveromyces lactis*, *Candida albicans*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe* were aligned using CLUSTAL 2.1 (Chenna et al. 2003) http://www.ebi.ac.uk/Tools/msa/clustalw2/). "M'/C' junction" indicates the location chosen for insertion of the fluorescent tag. "*Sp* truncation" indicates the start of the N-terminally truncated fragment used to crystallize *S. pombe* Sup35p (Kong et al. 2004), with "*Sp* structure" indicating where structure is first observed. "C domain start" and boxed methionines indicate the traditionally defined start of the C domain.

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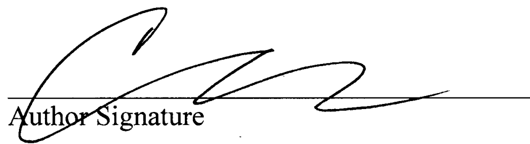

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