RESEARCH PAPERS

W8, a new Sup35 prion strain, transmits distinctive information with a conserved assembly scheme

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ABSTRACT. Prion strains are different self-propagating conformers of the same infectious protein. Three strains of the [PSI] prion, infectious forms of the yeast Sup35 protein, have been previously characterized in our laboratory. Here we report the discovery of a new [PSI] strain, named W8. We demonstrate its robust cellular propagation as well as the protein-only transmission. To reveal strain-specific sequence requirement, mutations that interfered with the propagation of W8 were identified by consecutive substitution of residues 5–55 of Sup35 by proline and insertion of glycine at alternate sites in this segment. Interestingly, propagating W8 with single mutations at residues 5–7 and around residue 43 caused the strain to transmute. In contrast to the assertion that [PSI] existed as a dynamic cloud of sub-structures, no random drift in transmission characteristics was detected in mitotically propagated W8 populations. Electron diffraction and mass-per-length measurements indicate that, similar to the 3 previously characterized strains, W8 fibers are composed of about 1 prion molecule per 4.7-A cross-β repeat period. Thus differently folded single Sup35 molecules, not dimeric and trimeric assemblies, form the basic repeating units to build the 4 [PSI] strains.

KEYWORDS. amyloid, prion strain, [PSI+], SUP35, yeast

ABBREVIATIONS. GFP, green fluorescent protein; PrP, prion protein; Aβ, amyloid β-protein; STEM, scanning transmission electron microscopy; mpl, mass per length; YPD, yeast extract, peptone, dextrose; SC, synthetic complete; 5-FOA, 5-fluoroorotic acid

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Received December 16, 2014; Revised March 15, 2015; Accepted April 2, 2015.
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**INTRODUCTION**

Prions are infectious proteins.\(^1\)\(^2\) A prion arises via misfolding and aggregation of a functional protein, and perpetuates itself by converting the normal protein into its like. Prion strains refer to different self-propagating structures of the same protein, which can differentially interact with the host to impart strain-specific phenotypes. Based on the disease phenotype, many mammalian PrP\(^\text{Sc}\) strains have been reported in laboratory mice, suggesting an equal number of distinct conformations.\(^3\) It is not completely understood how the simple protein sequence supports so many self-propagating conformers. While post-translational modifications increase molecular varieties, there was also suggestion that small biological molecules, such as lipids and nucleic acids, might assist the protein to maintain certain nucleating conformations.\(^4\)\(^–\)\(^6\)

Protein alone, however, is sufficient to support 3 strains of the yeast [\(\text{PSI}\)] prion, of which the specific infectivity was successfully propagated *in vitro* with purified recombinant protein.\(^7\)\(^–\)\(^9\) [\(\text{PSI}\)] strains are aggregates of the yeast Sup35 protein. In the normal conformation, Sup35 and Sup45 form the functional complex to terminate protein synthesis at the stop codon. When forming prion, Sup35 molecules are sequestered in the aggregates, causing read-through at stop codons and hence the suppression of nonsense mutations (nonsense suppression).\(^2\) Based on the strength of nonsense suppression, [\(\text{PSI}\)] strains are often classified as “strong” and “weak.”\(^1\)\(^0\) The strong-weak distinction facilitates experiments practically but lacks the resolution to uniquely define a [\(\text{PSI}\)] strain. More refined strain-typing methods revealed that distinct [\(\text{PSI}\)] strains could exhibit near identical strength in nonsense suppression.\(^1\)\(^1\)\(^,\)\(^1\)\(^2\) With the help of such methods, we have previously isolated and characterized 3 [\(\text{PSI}\)] strains, named VH, VK, and VL. VH exhibits strong nonsense suppression and VK and VL are weak strains.\(^1\)\(^1\) It is not yet clear how many different prion strains can be derived from the wild type Sup35 protein: [\(\text{PSI}\)] strains have been isolated in many laboratories, but it would require collaborative efforts in the future to thoroughly characterize the isolates, to compare and relate them.\(^1\)\(^0\)\(^–\)\(^2\)\(^1\) Nevertheless there is evidence to indicate the existence of more than 10 wild type [\(\text{PSI}\)] strains (unpublished). True and coworkers have suggested that, in the case of another yeast prion [\(\text{PIN}\)], strain variations could reach an astonishing number of 40.\(^2\)\(^2\)

Prion strains are amyloid polymorphs. Amyloid is a class of ordered protein aggregates where polypeptides adopt a \(\beta\)-rich structure to assemble linearly as a fiber.\(^2\)\(^3\)\(^–\)\(^2\)\(^5\) In the aggregates, the \(\beta\)-strands run perpendicular to the fiber axis and are connected axially by main-chain hydrogen bonds with the characteristic 4.7 Å inter-strand distance. Our previous electron diffraction and mass-per-length measurements revealed that 3 [\(\text{PSI}\)] strains, VH, VK, and VL, all contained near one Sup35 molecule per every 4.7 Å structural repeat.\(^2\)\(^6\) In contrast, in the case of \(\beta\)-amyloid aggregates, 2 different types of aggregates were observed to use dimers and trimers of A\(\beta\) peptides, respectively, as the basic building unit.\(^2\)\(^7\)\(^,\)\(^2\)\(^8\) The complexity of amyloid assemblies goes further. For example, the Het-s prion of the filamentous fungus *Podospora anserina* is assembled from a protein containing two 21 amino-acid tandem pseudo-repeats. In the fiber, each repeat forms a triangle-shaped structure with a height of 4.7 Å, so that a single protein molecule contributes to 9.4 Å fiber length;\(^2\)\(^9\) in fibers of a poly-glutamine peptide, electron microscopic measurements suggested that there was a simple, fractional number of 4/3 peptide for every 4.7 Å length;\(^3\)\(^0\) at a higher structural level, several single fibers can sometimes intertwine to form a multi-stranded species.\(^2\)\(^3\)\(^,\)\(^3\)\(^1\) It is not clear whether prion strain diversity mirrors the complexity of amyloid structures at all levels.

The [\(\text{PSI}\)] strains, with well developed genetic and biochemical assays, can yield further insight about structural variation of prions and amyloids. The first step for such investigation is to collect and characterize as many [\(\text{PSI}\)] strains as possible. Here we report the serendipitous discovery of a new...
results

isolation of the novel W8 strain

W8 arose in an experiment where multiple copies of Sup35 fragments were linked serially and incubated to form amyloid fibers spontaneously. When the yeast 5V-H19 [psi−] background32 was transformed with fibers of the Sup(1-40)-Gly15-Sup(1-40)-Sup(124-253) construct (containing 2 copies of the first 40 amino-acid residues of Sup35, linked by a spacer of 15 glycine residues, followed with residues 124 to 253 of Sup35 to enhance solubility), 2 of 10 [PSI+] transformants exhibited a strong nonsense suppression phenotype which was different from the rest, determined later to be of the VK strain (2W8, 8VK/174 LeuC colonies; yeast was transformed with protein fibers and the YCp111 plasmid which carried a complementary LEU2 marker, see Materials and Methods). The two “strong” isolates displayed a distinct, previously unobserved labeling pattern when a panel of Sup(1-61)-GFP constructs containing single or double mutations on the Sup(1-61) moiety were introduced into the cell (Fig. 1). The distinct phenotype was due to a new [PSI+] strain, rather than caused by random mutations or varied physiological responses in the yeast background: Sup(1-80)-GFP with a C-terminal Strep (II) affinity tag was overexpressed in the 2 isolates to facilitate purification of labeled prion particles by StrepTactin affinity chromatography.33 The purified yeast particles were introduced into freshly streaked 5V-H19 [psi−] cells. All of the resultant [PSI+] transformants exhibited the same nonsense suppression strength and the identical GFP-labeling pattern as the original W8 isolates (94/224; 106/217). The particles were also introduced into the 74-D-694 yeast genetic background;34 the resulting [PSI+] transformants again faithfully exhibited the same GFP-labeling pattern of W8 (25/96).

The discovery of W8 seemed fortuitous. Fibers of Sup(1-40)-Gly15-Sup(1-40)-Sup(124-253) were prepared anew and introduced into yeast: all [PSI+] transformants were of the VK type now, not W8 (18VK/224). Direct overexpression of the fusion protein in yeast (PIN+) via a multi-copy plasmid likewise only induced VK colonies de novo (28 VK/110 colonies; 20 VK, 1 VH/116). In addition, obviously, we had never observed the induction of W8 in the past by overexpressing in yeast either the full length Sup35 or its prion-inducing domain (1–123).

protein-only propagation of W8

The yeast particles were diluted in solution of highly purified Sup(1-80)-GFP, prepared from E. coli, to nucleate W8 fibers at 22°C quiescently. Numerous fibers were observed by fluorescence microscopy after 48 hours, whereas no aggregates were seen in protein solutions mock-seeded with buffer only. Fibers were fragmented by sonication and used to transform [psi−] cells. Compared to similarly diluted seeds, which caused very few [PSI+] transformants, the seeded solution resulted in large numbers of W8 transformants (Table 1). W8 infectivity was thus successfully propagated in a pure protein solution.

Genetic properties of W8

Strain competition

Haploid cells carrying W8 were crossed with cells harboring a different [PSI] strain. When the resultant diploid divided to form a colony, only one strain remained. For each competition, 16 randomly selected diploid colonies (from 2 independent crosses) were strain-typed, yielding consistent readings (16/16): VH dominated over W8, which in turn dominated over VK and VL (Fig. 2A). The relationship holds for both the 5V-H19 and the 74-D-694 backgrounds (16/16 each, Fig. 2B). The order of strain dominance seems to correlate with the strength of nonsense suppression (compare colony colors in Fig. 1A, the first row. The dominance relationship for the 5V-H19 background is VH>W8>VK>VL).
W8 Is Compatible with the PNM2 Allele

PNM2 is a mutant SUP35 allele encoding the Sup35(G58D) mutation. It has played significant roles in helping reveal the nature of [PSI]. The exact molecular mechanism of strong strain curing by PNM2 was recently under dispute, where 2 different scenarios were proposed, based on different experimental data. We tested if the PNM2 allele could support the propagation of W8. Yeast carrying W8 (5V-H19 [psi−] SUP35 his− LEU+ MATa) was mixed in rich liquid media with a [psi+] derivative of the opposite mating type, of which the wild type SUP35 allele was changed to PNM2 (5V-H19 [psi−] [pin−] PNM2 HIS+ leu− MATa). The mix was streaked on solid agar plates to select diploids by complementation of nutritional markers. All diploids remained [PSI+] as judged by the white colony color (due to nonsense suppression of the ade2-1 allele). Two random diploid colonies were further incubated to undergo meiosis to isolate spores in bulk. When spread on rich agar plates, none of the spore colonies were red, indicating the lack of [PSI] curing. Nevertheless the spores could be divided, based on colony colors, into 2 groups. The group of white colonies inherited the wild type SUP35; the group of pink colonies inherited the PNM2 allele (7/7 tested each, colonies were strain-typed to

| Experiment | Buffer alone | Protein alone | Seeds alone | Protein+Seeds |
|------------|--------------|---------------|-------------|---------------|
| I          | 0/224        | 0/224         | 0/224       | 28W8/224      |
| II         | 0/224        | 0/224         | 1W8/224     | 90W8/224      |

To nucleate prion fibers, W8 particles purified from yeast are added to Sup(1-80)-GFP purified from E. coli. Fibers are fragmented by sonic disruption and introduced into yeast cells. Nucleated fibers (Protein+Seeds) are much more infectious than similarly diluted yeast particles (seeds alone). Protein solutions mock-seeded by buffer (protein alone) have no infectivity. 28W8/224: 28 transformed yeast of the W8 strain type out of 224 randomly selected yeast colonies co-transformed with the YCp111 plasmid.
harbor W8). The mitotic stability of W8 was tested further: 3 spore colonies were randomly selected from each color group. They were grown in rich liquid media until saturation and then re-spread on agar plates to isolate single colonies again. None of the single colonies (>200 per plate) were red or contained any red sectors; and all colonies had the same color as their mitotic progenitors (Fig. S1). We hence concluded that PNM2 could indeed support the stable propagation of W8. Identical experiments were performed with the 74-D-694 background to reach the same conclusion.

No Evidence to Indicate That W8 Exists as a Dynamic Cloud of Sub-Structures

Bateman and Wickner demonstrated that a single [PSI+] isolate contained dynamic, inter-converting subpopulations of prions which could be distinguished by their differential transmission efficiency to 3 naturally occurring SUP35 alleles: Ref, the reference sequence, found in most laboratory yeast backgrounds; ΔJ9, containing deletion of 19 amino-acid residues (66-84) and a G162D change; and E9, containing 6 single substitutions (N109S, G162D, D169E, P186A, T206K, and H225D). We tested whether W8 was a dynamic ensemble of sub-structures using the same cytoduction recipients kindly provided by the original authors (see Materials and Methods for a brief introduction of cytoduction). The W8 strain maintained in 2 independent 74-D-694 colonies were first confirmed to transmit well to yeast of all 3 SUP35 genotypes (type D according to ref. 37). The two colonies were then grown in 3 ml rich liquid media at 30°C for 24 hours until late log phase. Ten microliters of each culture were transferred to fresh media and grew for another 24 hours. The procedure was repeated daily and the cultures of the 7th day were spread on rich agar plates to isolate 8 independent colonies (4 each) for cytoduction experiments. All of the random isolates maintained the good transmission efficiency to all 3 SUP35 sequences (Table 2A). The whole experiment was repeated with another 2 new W8 colonies. Eight random colonies each from cultures of
TABLE 2(A). Cytoduction of W8 populations

| Donor     | Recipient allele | [PSI+] | Total (ρ+) | %  | Transmission Type |
|-----------|------------------|--------|------------|----|-------------------|
| W8-1      | Reference        | 49     | 49         | 100|                   |
|           | Δ19              | 55     | 56         | 98 | D                 |
|           | E9               | 70     | 70         | 100|                   |
| W8-2      | Reference        | 54     | 55         | 98 | D                 |
|           | Δ19              | 54     | 55         | 98 | D                 |
|           | E9               | 78     | 79         | 99 |                   |
| W8-3      | Reference        | 56     | 56         | 100|                   |
|           | Δ19              | 54     | 56         | 96 | D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-4      | Reference        | 56     | 56         | 100|                   |
|           | Δ19              | 56     | 56         | 100| D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-1-1    | Reference        | 56     | 56         | 100|                   |
|           | Δ19              | 55     | 56         | 98 | D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-1-2    | Reference        | 54     | 56         | 96 | D                 |
|           | Δ19              | 54     | 56         | 96 | D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-1-3    | Reference        | 55     | 55         | 100|                   |
|           | Δ19              | 53     | 56         | 95 | D                 |
|           | E9               | 55      | 55        | 100|                   |
| W8-1-4    | Reference        | 54     | 54         | 100|                   |
|           | Δ19              | 54     | 56         | 96 | D                 |
|           | E9               | 53     | 56         | 95 |                   |
| W8-2-1    | Reference        | 55     | 56         | 98 | D                 |
|           | Δ19              | 56     | 56         | 100| D                 |
|           | E9               | 55     | 55         | 100|                   |
| W8-2-2    | Reference        | 55     | 55         | 100|                   |
|           | Δ19              | 53     | 54         | 98 | D                 |
|           | E9               | 47     | 47         | 100|                   |
| W8-2-3    | Reference        | 56     | 56         | 100|                   |
|           | Δ19              | 55     | 56         | 98 | D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-2-4    | Reference        | 54     | 56         | 96 | D                 |
|           | Δ19              | 54     | 56         | 96 | D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-3-1    | Reference        | 52     | 53         | 98 | D                 |
|           | Δ19              | 54     | 54         | 98 | D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-3-2    | Reference        | 54     | 55         | 98 | D                 |
|           | Δ19              | 53     | 53         | 100| D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-3-3    | Reference        | 55     | 56         | 98 | D                 |
|           | Δ19              | 54     | 55         | 98 | D                 |
|           | E9               | 55     | 56         | 98 | D                 |
| W8-3-4    | Reference        | 55     | 56         | 98 | D                 |
|           | Δ19              | 52     | 55         | 95 | D                 |
|           | E9               | 48     | 51         | 94 |                   |
| W8-4-1    | Reference        | 54     | 55         | 98 | D                 |
|           | Δ19              | 55     | 55         | 100| D                 |
|           | E9               | 56     | 56         | 100|                   |
| W8-4-2    | Reference        | 53     | 54         | 98 | D                 |
|           | Δ19              | 52     | 52         | 100| D                 |
|           | E9               | 54     | 55         | 98 |                   |
| W8-4-3    | Reference        | 54     | 54         | 100|                   |
|           | Δ19              | 50     | 55         | 91 | D                 |

(Continued on next page)
day 7 and of day 14 were assayed. Again, all colonies transmitted W8 to the 3 SUP35 genotypes with near 100% efficiency (Table 2A).

W8 therefore did not change transmission characteristics during continuous growth of the host—there is no indication for the existence of dynamic W8 subpopulations. We performed identical experiments with the VH strain. No drift in transmission profile was detected. All random colonies—16 from day 7 cultures, 8 from day 14’s—transmitted VH well to the Ref and D19 backgrounds but none transmitted the prion to the E9 background (Type C, Table 2B). Further experiments with the VK and VL strains revealed similar unvaried transmission profiles among all random colonies (both Type C; Table S1; the lower expression level of E9, which supported W8, might exacerbate the transmission barrier for the VH, VK, and VL strains. See Materials and Methods).

### STRAIN-SPECIFIC SEQUENCE

#### The Minimal Infectious Fragments

The W8 particles were purified from yeast to nucleate amyloid fibers in pure solutions of various Sup35 N-terminal fragments, which were fused with GFP to enhance protein solubility and facilitate experimental observation. Numerous fibers were observed after 48-hour quiescent incubation at 22°C. They were fragmented by sonic disruption and introduced into yeast. Compared with diluted seeds, Sup(1-53)- and Sup(1-61)-GFP fibers exhibited increased infectivity (11W8/224 and 16W8/224, respectively; diluted seeds: 2W8/224; protein alone: 0/224 and 0/224), but Sup(1-40)- and Sup(1-47)-GFP fibers were not infectious (1W8/224 and 1W8/224, respectively).

More stringent genetic experiments revealed that although Sup(1-61)-GFP supported the
TABLE 2(B). Cytoduction of VH populations

| Donor | Recipient Allele | \([PSI^+]\) | Total \((\mu^+)\) | % | Transmission Type |
|-------|------------------|------------|---------------|---|------------------|
| VH-1  | Reference        | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 54         | 56            | 96  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-2  | Reference        | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 52         | 56            | 93  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-3  | Reference        | 54         | 54            | 100 |                  |
|       | \(\Delta 19\)    | 51         | 56            | 91  | C                |
|       | E9               | 0          | 50            | 0   |                  |
| VH-4  | Reference        | 51         | 51            | 100 |                  |
|       | \(\Delta 19\)    | 56         | 56            | 100 | C                |
|       | E9               | 0          | 50            | 0   |                  |
| VH-1-1 (after 7d) | Reference | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 49         | 56            | 88  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-1-2 | Reference   | 55         | 55            | 100 |                  |
|       | \(\Delta 19\)    | 53         | 56            | 95  | C                |
|       | E9               | 0          | 55            | 0   |                  |
| VH-1-3 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 54         | 54            | 100 | C                |
|       | E9               | 0          | 55            | 0   |                  |
| VH-1-4 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 56         | 56            | 100 | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-2-1 (after 7d) | Reference | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 50         | 55            | 91  | C                |
|       | E9               | 0          | 55            | 0   |                  |
| VH-2-2 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 48         | 52            | 92  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-2-3 | Reference   | 55         | 55            | 100 |                  |
|       | \(\Delta 19\)    | 52         | 53            | 98  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-2-4 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 55         | 56            | 98  | C                |
|       | E9               | 0          | 53            | 0   |                  |
| VH-3-1 (after 7d) | Reference | 52         | 56            | 93  | C                |
|       | \(\Delta 19\)    | 51         | 56            | 91  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-3-2 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 52         | 56            | 93  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-3-3 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 56         | 56            | 100 | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-3-4 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 54         | 56            | 96  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-4-1 (after 7d) | Reference | 53         | 53            | 100 |                  |
|       | \(\Delta 19\)    | 49         | 54            | 91  | C                |
|       | E9               | 0          | 53            | 0   |                  |
| VH-4-2 | Reference   | 56         | 56            | 100 |                  |
|       | \(\Delta 19\)    | 49         | 51            | 96  | C                |
|       | E9               | 0          | 56            | 0   |                  |
| VH-4-3 | Reference   | 55         | 55            | 100 |                  |
|       | \(\Delta 19\)    | 54         | 56            | 96  | C                |
|       | E9               | 0          | 56            | 0   |                  |

(Continued on next page)
propagation of W8 in vitro, it did so with relaxed fidelity in vivo. Overexpression of Sup(1-61)-GFP in yeast ([W8], [pin\(^{-}\)]) caused prion strain mutation in about 3% of the cells (W8 to VK), as well as [PSI] curing in about 40% of the cells (Table 3; [PSI] curing can be thought as a kind of strain mutation where W8 changed to a non-infectious conformation). The occurrence of strain mutation and curing were reduced to about 5% with Sup(1-80)-GFP overexpression and were completely abolished with Sup(1-114)-GFP and the full-length Sup35 (Table 3). The data suggested that some extra sequence beyond the first 61 amino-acid residues was required to protect the core structure of W8 from mis-templating. We note that a similar phenomenon was also observed for the VL strain.8,12

Strain-Specific Sequence

We propagated W8 in 2 sets of yeast backgrounds containing single mutations on the chromosomal SUP35 allele: the first set consisted of 51 mutations, changing amino-acid residues 5-55 to proline one by one; in the second set (26 mutations), a glycine residue was inserted in front of every other amino acid from position 5 to 55.38 Both kinds of changes could disrupt the \(\beta\)-structure. The W8 strain was introduced into the mutant backgrounds by mating with the [psi\(^{-}\)] cells that contained the mutations. With the exception of G40P, G47P, N48P, and Gi39 (indicating glycine insertion in front of residue 39), all resultant heterozygotes remained [PSI\(^{+}\)]. To determine whether mutant alleles alone could support the propagation of W8, we randomly picked 2 colonies each, grew

| Donor | Recipient Allele | \([\text{PSI}^+]\) | Total (\(\mu^{+}\)) | % | Transmission Type |
|-------|-----------------|----------------|-----------------|---|-----------------|
| VH-4-4 | Reference | 51 | 51 | 100 | C |
| \(\Delta 19\) | 51 | 54 | 94 | C |
| E9 | 0 | 55 | 0 | |
| VH-3-1 (after 14d) | Reference | 56 | 56 | 100 | C |
| \(\Delta 19\) | 55 | 56 | 98 | |
| E9 | 0 | 56 | 0 | |
| VH-3-2 | Reference | 56 | 56 | 100 | C |
| \(\Delta 19\) | 55 | 55 | 100 | |
| E9 | 0 | 56 | 0 | |
| VH-3-3 | Reference | 56 | 56 | 100 | C |
| \(\Delta 19\) | 49 | 56 | 88 | |
| E9 | 0 | 55 | 0 | |
| VH-3-4 | Reference | 56 | 56 | 100 | C |
| \(\Delta 19\) | 47 | 56 | 84 | |
| E9 | 0 | 56 | 0 | |
| VH-4-1 (after 14d) | Reference | 56 | 56 | 100 | C |
| \(\Delta 19\) | 52 | 56 | 96 | |
| E9 | 0 | 56 | 0 | |
| VH-4-2 | Reference | 47 | 47 | 100 | C |
| \(\Delta 19\) | 50 | 52 | 96 | |
| E9 | 0 | 52 | 0 | |
| VH-4-3 | Reference | 54 | 56 | 96 | C |
| \(\Delta 19\) | 45 | 51 | 88 | |
| E9 | 0 | 56 | 0 | |
| VH-4-4 | Reference | 47 | 47 | 100 | C |
| \(\Delta 19\) | 38 | 39 | 97 | |
| E9 | 0 | 56 | 0 | |

\([\text{PSI}]\) particles together with healthy mitochondria (\(\mu^{+}\)) are transmitted from 4 founder colonies (i.e., W8-1 to W8-4 or VH-1 to VH-4) to recipients with different SUP35 alleles (Reference, \(\Delta 19\), and E9). The 4 founders were continuously grown for 7 or 14 days, and the cultures were spread on YPD plates to randomly isolate 4 colonies each (e.g., W8-1-1 to W8-1-4 from the founder W8-1) for further cytoduction analysis. Percentages of \([\text{PSI}^+]\) colonies in all \(\mu^{+}\) cytoductants are calculated. Transmission type is defined according to ref. 37. Type C — transmitting well (\(>25\%\)) to Reference and \(\Delta 19\), but poorly to E9; type D — transmitting well to all 3.

The data suggested that some extra sequence beyond the first 61 amino-acid residues was required to protect the core structure of W8 from mis-templating. We note that a similar phenomenon was also observed for the VL strain.8,12

TABLE 2(B). Cytoduction of VH populations (Continued)
them in sporulation media to undergo meiosis (thus segregating \(SUP35\) alleles), collected haploid spores in bulk, and then checked the strain type of mutant spores. The analysis revealed that only 12 proline substitutions and 8 glycine insertions could still support W8 propagation. Their unique distribution along the Sup35 sequence suggested a distinct Sup35 fold (Fig. 3).

**Relationship with the VK Strain**

Instead of curing \([\psi^+]\), 8 incompatible mutations—N5P, Q6P, G7P, G43P, Gi7, Gi9, Gi43, and Gi45—caused W8 to VK transmutation (Fig. 3). When the heterozygotes of these mutants were sporulated, 3 types of colonies were observed: (1) white W8 colonies, all of which inherited the wild type \(SUP35\) allele; (2) pink VK colonies, most of which inherited the mutant allele; and (3) red \([\psi^+]\) colonies, nearly all of which inherited the mutant allele (Table S2). To provide further support, the W8 strain was transmitted directly to Q6P and G43P backgrounds by cytoduction. All resultant \([\psi^+]\) cytoductants exhibited the specific GFP-labeling pattern of VK, not W8. In addition, there were mild transmission barriers as judged by the appearance of a moderate proportion of \([\psi^+]\) cytoductants (2 biological repeats. Q6P: 21VK, 35[\psi^+] /56 and 40VK, 16[\psi^+] /56. G43P: 38VK, 17[\psi^+] /55 and 50VK, 6[\psi^+] /56). In contrast, W8 was faithfully transmitted to the wild type control without impediment (55W8/55 and 55W8/55). When VK particles composed of the mutant Q6P and G43P proteins were cytoduced back to the 74-D-694 background which expressed the wild type Sup35 protein, all \([\psi^+]\) cytoductants remained of the VK strain type (2 biological repeats. Q6P: 47VK/47 and 22VK, 3[\psi^+] /25. G43P: 46VK, 2[\psi^+] /48 and 32VK, 4[\psi^+] /36).

The VK strain doesn’t easily transmute to W8. When the VK strain was analogously transmitted to the 8 mutants, all spores faithfully propagated VK—no reversion to W8 (Fig. 3). Direct transmission of VK to Q6P and G43P backgrounds by cytoduction likewise did not cause any change in strain type (2 biological repeats. Q6P: 31VK, 24[\psi^+] /55 and 34VK, 20[\psi^+] /54. G43P: 49VK, 1[\psi^+] /50 and 46VK, 1[\psi^+] /47. Wild type control: 47VK, 9[\psi^+] /56 and 47VK, 7[\psi^+] /54). Unsurprisingly, the VK strain type was maintained when the Q6P and G43P prions were subsequently re-propagated with the wild-type Sup35 protein (reverse cytoduction, 2 biological repeats. Q6P: 26VK, 1[\psi^+] /27 and 46VK, 8[\psi^+] /54. G43P: 42VK, 4[\psi^+] /46 and 30VK/30. Wild type control: 22VK, 3[\psi^+] /25 and 35VK, 8[\psi^+] /43).

We next tested if W8 could be induced from VK under stronger conditions. Seven chromosomal mutations—Q15P, N21P, Y35P, A37P, Gi21, Gi23, and Gi35—cured VK but supported the propagation of W8 (Fig. 3). We propagated VK to cells overexpressing the mutants and determined whether W8 was induced via mis-nucleation. The experiments were carried out in 4 steps: (1) we prepared a 74-D694 \([\psi^+]\) derivative of which the chromosomal \(SUP35\) gene was deleted, and the VK strain was maintained by a copy of the wild type \(SUP35\) gene carried on a centromere-based plasmid; (2) the plasmid was replaced with a multi-copy plasmid which overexpressed one of the mutants. Four independent colonies that remained \([\psi^+]\) were selected after the plasmid shuffle; (3) the multi-copy plasmid in the \([\psi^+]\) colonies was replaced again with a centromere-based plasmid, which expressed the wild type protein from the native

| W8 (%) | VK (%) | \(\psi^+\) (%) | Total |
|--------|--------|----------------|-------|
| Vector | 224 (100) | 0 (0) | 0 (0) | 224 |
| Sup(1-61)-GFP | 62 (53.4) | 4 (3.4) | 50 (43.1) | 116 |
| Sup(1-70)-GFP | 65 (57.0) | 3 (2.6) | 46 (40.4) | 114 |
| Sup(1-80)-GFP | 77 (68.8) | 15 (13.4) | 20 (17.9) | 112 |
| Sup(1-114)-GFP | 79 (69.3) | 6 (5.3) | 29 (25.4) | 114 |
| Sup35 FL | 105 (93.8) | 0 (0) | 7 (6.3) | 112 |
| 110 (98.2) | 1 (0.9) | 1 (0.9) | 112 |
| Sup(1-100)-GFP | 224 (100) | 0 (0) | 0 (0) | 224 |

The W8 strain is propagated in yeast overexpressing various Sup35 fragments (left). Randomly selected colonies are strain-typed. FL: full-length.
promoter; (4) the resulting \([\text{PSI}^+]\) cells were strain-typed. Table 4 shows that most colonies maintain the VK strain type after the passages. Among more than 240 strain-typed colonies, W8 appears only twice, along with 3 VH and 59 VK colonies, in the propagation with the Gi23 mutant. The near absence of W8 suggested that the VK structure could not readily morph into W8. As W8 dominated over VK in strain competition, its induction in the cell
would have led to quick establishment and subsequent detection.

**FIBER ASSEMBLY**

Electron diffraction of ice-embedded Sup(1-80)-GFP fibers nucleated by yeast W8 particles showed the 4.7 Å spacing characteristic of the cross-β structure (Fig. 4A; see Fig. S2 for the similar diffraction pattern of Sup(1-61)-GFP fibers). We carried out mass-per-length (mpl) measurements of infectious Sup(1-61)-GFP and Sup(1-80)-GFP fibers. Freeze-dried samples were imaged by scanning transmission electron microscopy (STEM) and comparable proportions of fibers with distinguishable morphologies, classified as lanky, wavy and jaggy, were observed in the Sup(1-61)-GFP sample. The mpl measurements showed that there was about one Sup(1-61)-GFP molecule per 4.7 Å repeat length regardless of fiber morphology (measured value (all morphologies together) = 7.93 ± 0.56 kDa/Å, theoretical value = 7.79 kDa/Å; Fig. 4B). The Sup(1-80)-GFP fibers were significantly more homogeneous, composed mainly of lanky fibers (Fig. 4C). This is in accordance with reduced W8 curing when Sup(1-80)-GFP was overexpressed in yeast (described above). The good accordance and strong infectivity of Sup(1-80)-GFP fibers (Table 1) suggested that the predominant lanky fibers were the infectious species. We however could not rule out the possibility that the minor wavy and jaggy fibers also contributed to the infectivity of Sup(1-80)-GFP samples. The mpl measurement again revealed that there was about one Sup(1-80)-GFP molecule for each 4.7 Å cross-β repeat (measured value = 8.50 ± 0.65 kDa/Å, theoretical value = 8.26 kDa/Å; Fig. 4C).

The simplest architecture of the W8 strain, consistent with the data, would have Sup35 monomers stacking up with a 4.7 Å periodicity to assemble an infectious fiber. Our previous data on the VH, VK, and VL strains suggested the same molecular arrangement. The strain characteristics of VH, VK, VL and W8 then must be specified by 4 distinct, single-chain folding patterns of Sup35.

| Table 4. Forced propagation of VK |
|----------------------------------|
| **Forward barrier** | **Reverse barrier** | **Colony 1-1** | **Colony 1-2** | **Colony 2-1** | **Colony 2-2** | **Total** |
| Q15P | ++ | ++ | 1K, 2L, 1K*, 1[psi+] | 2L, 3K* | 6K* | 6K* | 1K, 4L, 16K*, 1[psi+] | (22) |
| N21P | + | ± | 6K | 12K | 6K | 6K | 30K (30) |
| Y35P | ++ | − | 6K | N/A | 6K | 6K | 18K (18) |
| A37P | ± | ± | 5K | 5K | 6K | 16K | 32K (32) |
| G21 | ++ | ± | 19K | 11K | 6K | 12K | 48K (48) |
| Gi23 | ++ | + | 2H, 3K | 1H, 10K, 1W | 22K, 1W | 24K | 3H, 59K, 2W (64) |
| Gi35 | − | + | 5K | 5H, 1K, 7K*, 1[psi+] | 3H, 4K, 5K* | 6H, 15K, 12K*, 1[psi+] | (34) |
| WT | − | − | 7K | 7K | 6K | 27K (27) |

Experiments are performed by plasmid shuffle. The VK strain is propagated in a 74-D-694 derivative where SUP35 is relocated to a centromere-based (CEN) plasmid with a URA3 selection marker (plasmid: wt1). Yeast is transformed with a 2μ-based plasmid overexpressing the mutant (plasmids in cell: wt1 + mut1), replicated on 5-FOA plates to lose the CEN plasmid (mut1), transformed with another 2μ-based plasmids with a URA3 marker but the same mutant (mut1 + 2), to lose the first plasmid (remaining plasmid: mut2), transformed with a CEN plasmid carrying the wild type SUP35 (mut2 + wt2), and finally to lose the second mutant plasmid on 5-FOA replica plates (wt2). The degree of [PSI] curing is estimated visually (++) by observing the appearance of red colony/sectors between transmission stages (wt1 + mut1) (forward barrier) and (mut2 + wt2) (reverse barrier). Four [PSI+] colonies derived from 2 independent starting colonies are selected at the (mut1) stage (colonies 1-1, 1-2, 2-1, and 2-2). Five to 24 downstream derivatives each are randomly selected at the (mut2 + wt2) stage, carried through the rest of the experiment, and strain-typed. H = VH, K = VK, L = VL, W = W8. K* indicates temporary co-existence of VK and VL in the cell, which, for the 74-D-694 background, could last for more than 200 cell generations before VL wins out completely (unpublished).
FIGURE 4. Diffraction and mass-per-length (mpl) measurements of W8 fibers. (A) Electron diffraction pattern of ice-embedded, unoriented Sup(1-80)-GFP(+36) fibers. The 4.7 Å reflection, the water (vitreous ice) ring (≈3.6 Å), and gold internal reference (2.3 Å) are indicated. GFP(+36) is an engineered GFP containing 29 extra positively charged residues which enhances sample adsorption and thus the diffraction signal. The same diffraction pattern was obtained from Sup(1-61)-GFP and Sup(1-80)-GFP fibers which are constructed with the commonly used eGFP (see Fig. S2). (B & C) Histograms of mpl measurements from STEM images of freeze-dried Sup(1-61)-GFP and Sup(1-80)-GFP fibers, respectively. Visually distinguished morphologies, classified as lanky, wavy, and jaggy, are color-coded (as labeled). Tobacco mosaic virus (TMV) is used as internal calibration standard. One prion molecule per 4.7-Å repeat corresponds to 7.79 kDa/Å for Sup(1-61)-GFP and 8.26 kDa/Å for Sup(1-80)-GFP, as marked. Bar = 500 Å.
strains. For example, Sharma and Liebman described an unspecified [PSI] isolate that continuously gave off stable strong and weak strains;\textsuperscript{40} McGlinchey et al. reported the suicidal [PSI], which strongly titrated out the functional Sup35 protein and consequently killed the host.\textsuperscript{41} While several additional “subtle” [PSI] strains (i.e. weak or slightly transmutable but unambiguously strain-typed; unpublished) were found, we had not expected W8, with its robust propagation properties. It is now hopeful that other robust [PSI] strains will be discovered.

The W8 strain changed to the VK strain if propagated by mutant Sup35 proteins with single proline substitution at residues 5, 6, 7 and 43 and glycine insertion in front of residues 7, 9, 43, and 45. The residues might be critically involved in maintaining the unique W8 structure, whose disruption by mutation could open up the chain and expose a protected structural element to mis-nucleate the VK structure. VK, however, is unlikely just a degenerated core of W8, as it does not readily transmute to W8, even under forced conditions. The similarity of the 2 structures may in effect end at the protected structural element proposed above.

The electron diffraction and mass-per-length measurements clearly established that there was one Sup35 molecule for each 4.7 Å/cross-b repeat in the W8 fiber. We favor the assembly model that Sup35 monomers, with the strain-specific fold, stack up along the fiber axis with a 4.7 Å periodicity. The model does not rule out the periodic arrangement where part of the polypeptide sequence, possibly in the terminal region, goes up a stack, and the lost mass is regained symmetrically from the molecule below. Based on solid-state NMR distance measurements, Gorkovskiy et al. recently provided strong evidence for the in-register parallel stacking of Sup35 molecules in an infectious amyloid sample.\textsuperscript{42–44} While the parallel in-register arrangement is perfectly consistent with our data and model and is likely the architecture of many if not all [PSI] strains, certain packing adjustments seem necessary to build a pronouncedly wavy fiber, such as the VK fiber.\textsuperscript{8,26,38} Further progress in [PSI] structures will require the application of high-resolution biophysical techniques on homogeneous preparations. In this regard, we showed here that sample homogeneity was greatly improved when infectious W8 fibers were prepared with longer Sup35 fragments. In addition, the suggestion that a single [PSI\textsuperscript{+}] isolate contains a dynamic “cloud” of structures has caused concerns regarding the fundamental limit on achievable sample quality. The fact that we did not see clouds suggested one might not need to worry. There may be prion strains that are intrinsically unvarying in folding or nearly so, such as W8, VH, VK, and VL and possibly many more.

\section*{MATERIALS AND METHODS}

\subsection*{Yeast Strains and General Methods}

Experiments were performed with the 5V-H19 (SUQ5 ade2-1(UAA) can1-100 leu2-3,112 ura3-52) and 74-D-694 backgrounds (ade1-14 (UGA) leu2 ura3 his3 trp1).\textsuperscript{32,34} The 4830 background was used for cytoduction experiments (see below).\textsuperscript{37,45} Standard protocols were used for media preparation and yeast genetic manipulation.\textsuperscript{46} Escherichia coli strains DH5\textalpha and BLR(DE3)pLysS (Novagen) were used for plasmid construction and protein expression, respectively.

\subsection*{[PSI] Strain Typing}

White, pink and dark pink colonies were selected for strain typing. Two sets of plasmids were used: the first set consisted of 5 plasmids—Ycp33-I-SUPF, YCp33-I-SUPF (G58D), YCp33-I-SUPF(G44R), YCp33-I-SUPF(S17R), and YCp33-I-SUPF(Q15R)—which expressed the full length Sup35 protein and single mutations from the native promoter. Their expression in yeast cells changed the colony color in a strain-specific manner; the second set consisted of 4 plasmids—YEp195-Cup1-Sup(1-61)-GFP, YEp195-Cup1-Sup (1-61)(G20D)-GFP, YEp195-Cup1-Sup(1-61) (Q23P)-GFP, and YEp195-Cup1-Sup(1-61) (Q23P, N27P)-GFP—which expressed GFP fusion proteins via a copper-inducible CUP1
promoter. They differentially labeled \([\text{PSI}^-]\) strains.47

**Protein Over-Expression in Yeast**

Different Sup35 fragments, sequences carried by the multi-copy plasmid YEp195 (URA3), were overexpressed from a 500bp CUP1 promoter. Freshly transformed yeast colonies were grown at 30°C in 3 ml synthetic complete media lacking uracil (SC-Ura) but containing 50 \(\mu\)M CuSO4. After 48 hours, the culture was spread on YPD plates. Colonies were randomly picked up, transferred onto new YPD plates, replicated on SC plates containing 50 mg/L uracil and 0.75g/L 5-fluoro-orotic acid (5-FOA) to counter-select cells that had lost the YEp195 plasmid, and then strain-typed. Each experiment was performed with at least 2 independent colonies.

**Purification of W8 Particles**

\([\text{W8}][\text{pin}^-]\) cells were transformed with the plasmid YEp195-Cup1-Sup(1-80)-GFP-Strep (II)-His6. Prion particles were isolated from the cells and purified by StrepTactin (IBA) affinity chromatography as described.33,47 Sup(1-80)-GFP labeled yeast particles were used for all experiments except experiments described in “minimal infectious fragments” and the mass-per-length measurement of Sup(1-61)-GFP fibers, which were nucleated from yeast particles labeled with Sup(1-61)-GFP-Strep(II).

**Recombinant Protein Preparation**

The E. coli strain BLR(DE3)pLysS was used for protein expression. Proteins were purified from E. coli extracts by Ni-NTA affinity columns (Qiagen) followed by StrepTactin affinity chromatography as described.33,47 Purified proteins were nucleated immediately.

**Fiber Formation and Infection**

Catalytic amount of purified W8 prion particles were added to 250 \(\mu\)l of purified recombinant proteins (100 \(\mu\)M) to nucleate fiber growth at 22°C for 48 hours without agitation. The reaction was sonicated on ice for 10 seconds with 6 W power output. Four microliters of the reaction were used for yeast transformation (described below). The transmants were strain-typed. For electron microscopic analysis, fibers were collected by ultracentrifugation (200,000×g, 2 hours) at 4°C without sonication.

**Yeast Transformation by \([\text{PSI}^-]\) Particles**

Spheroplasts (\([\text{psi}^-][\text{pin}^-]\)) were prepared as described.47 They were resuspended in Z1 buffer (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5 and 30 mM CaCl2) and aliquoted into 100 \(\mu\)l per reaction. Eight microliters of yeast prion particles or 4 \(\mu\)l of nucleated fibers were added to the spheroplasts to incubate at 22°C for 15 minutes, and 2 \(\mu\)l of YCp111 plasmid (1 mg/ml) were then added to incubate for 15 minutes, followed by the addition of 1 ml Z2 buffer (20% (w/v) polyethylene glycol 3350, 10 mM Tris-HCl pH 7.5 and 30 mM CaCl2) for another 15- minute incubation. Spheroplasts were collected at 2,100×g for 5 minutes, resuspended in 150 \(\mu\)l Z3 buffer (1 M sorbitol, 30 mM CaCl2, 1/3 strength YPAD, 33 mg/l leucine, 7 mg/l uracil, 7 mg/l histidine HCl and 7 mg/l tryptophan) and incubated at 30°C for more than 30 minutes. Four milliliters of warmed top agar (SC-Leu, 1.2 M sorbitol and 1.6% (g/ml) Bacto agar) were added to the cell suspensions and poured onto a sorbitol agar plate (SC-Leu, 1.2 M sorbitol, and 2% (g/ml) Bacto agar). The plate was incubated at 30°C for 3–5 days and colonies were transferred onto SC-Leu plates. \([\text{PSI}^+]\) transformants were identified by colony color and strain-typed.

**Strain Competition**

Strain competition was performed in both the 5V-H19 and 74-D-694 genetic background. Two yeast isolates bearing different prion strains were crossed (i.e., \([\text{MATa}][\text{YCp111}][\text{pin}^-][\text{PSF}^+]\times[\text{MATa}][\text{YCp33}][\text{pin}^-][\text{PSF}^-]\); a=(strain a) and b=(strain b)). Two independent mating reactions were performed for each strain pair. For example, for VH×W8, the 2
crosses were (MATα [VH]) × (MATα [W8]) and (MATα [W8]) × (MATα [VH]). Eight diploids from each cross were randomly selected from SC-Leu, Ura plates and strain-typed.

### Proline Substitution, Glycine Insertion, and PNM2

Chromosomal SUP35 proline substitutions, glycine insertions, and the PNM2 mutation were prepared as described.11,38 Two independently obtained mutant colonies (background: 5V-H19 [psi−]) were mated with 2 independent [W8] colonies (5V-H19 SUP35 (WT) ΔHIS4:kanMX [YCp111(LEU2)]) and heterozygotes selected on SC-His, Leu. Two random heterozygotes each were transformed with the YEpi195-Cup1-Sup(1-61)-GFP plasmid to observe GFP labeling. A mutant is classified as “[PSI] no more” (PNM) when less than 5% of the diploid cells (> 100 cells observed each), growing in liquid media, exhibited the particulate [PSI+] labeling. PNM’s were not analyzed further. Otherwise, heterozygotes were subjected to random sporulation for further analysis on YPD plates. Spores of each mutation were classified according to colony color (e.g. white, light pink, pink, dark pink, and red). The allele type of 8 colonies from each color class were determined by BamHI (proline and glycine mutations) or BstXI (PNM2) digestion of a genomic PCR fragment (SUP35(-1243-372)). Mutant alleles were distinguished by the presence of a BamHI restriction site before the start codon, or by the absence of the BstXI site, which was destroyed in the G58D construct.11 The [PSI] compatibility of each mutant was then scored according to colony color (WT color = 1; red = 0; whiter than WT >1; darker than WT <1). Mutations with scores ≠ 0 or 1 were further strain-typed by GFP labeling (2 independent spores each) and the SUP35 allele reconfirmed by sequencing. For PNM2 experiments with 74-D-694, the mutant colonies were transformed with pRS313 (HIS3) and then mate with [W8] colonies (74-D-694 SUP35(WT) his3 [YCp111(LEU2)]) to facilitate diploid selection on SC-His, Leu.

### Fluorescence Microscopy

Cells were grown to mid log phase in SC-Ura media, fixed with 3.7% (v/v) formaldehyde at 22°C for 30 minutes, and then observed with a customized Olympus IX-71 Inverted Microscope. Images were processed with the DeltaVision Core Live-cell Imaging System.

### Electron Microscopy and Diffraction

For negatively stained sample, specimens were prepared by placing 3 µl of appropriately diluted samples on a 300-mesh carbon-coated copper grid, and then stained with 5 drops of 1% (w/v) uranyl acetate after blotting. Images were collected at 11,000× and 26,000× magnification with a Tecnai G2 Spirit TWIN Transmission Electron Microscope (FEI) operating with an accelerating voltage of 80 keV. For electron diffraction, ice-embedded samples were prepared on Quantifoil 300-mesh copper grids (Jena, Germany) deposited with a thin film of gold as calibration standard. Images were obtained under low dose conditions using a 200-keV Tecnai F20 electron microscope (FEI) equipped with a 4k × 4k CCD camera (Gatan). The electron dose given to the specimen during electron diffraction was in the order of 30 e/A².

### Mass-Per-Length Measurements

Dark-field images of unstained, freeze-dried specimens were recorded at the STEM facility of Brookhaven National Laboratory, using a scanning pixel size of (20 Å)² as described.26 Tobacco mosaic virus (TMV) particles, which have the mpl measure of 13.1 kDa/Å, were added as the internal calibration standard. Measurements on the images were performed with the program PCMASS32.48 Unencumbered segments of fibers were selected in 560 A × 240 A (for Sup(1-61)-GFP) or 400 A × 240 A (Sup(1-80)-GFP) boxes. Fibers were classified according to visually distinguishable morphologies.
Seventy-two fibers—23 lanky fibers with 178 measurements (23 (178)), 23 (194) wavy and 26 (244) jaggy—were measured for the Sup(1-61)-GFP sample and 78 fibers—67 (553) lanky, 8 (56) wavy and 3 (31) jaggy—were measured for Sup(1-80)-GFP. Data were processed with the Origin 9 program. Histograms were produced with 0.25 kDa/A binning windows.

**Mitotic Propagation of [PSI] Strains**

The mutant ura3 allele of 74-D-694 was replaced with the wild type allele by homologous recombination to obtain 74-D-694 (URA3) derivatives (MATa URA3 ade1-14 (UGA) leu2 his3 trp1 [pin]) carrying various [PSI] strains: [psi−], [W8], [VH], [VK], and [VL]. For each strain type, 2 independently obtained URA3 derivatives were streak-purified 3 times on YPD plates to randomly select 2 founder colonies, which were subject to cytoduction analysis. They were also grown in 3 ml rich liquid media (YPAD) at 30°C for 24 hours till late log phase. Ten microliters of each culture were transferred to fresh media and grew for another 24 hours. The procedure was repeated daily and the culture of the 7th (or 14th) day was spread on YPD plates to randomly select 4 independent colonies from each founder. The mitotic progenies were subject to cytoduction analysis.

**The 4830 Genetic Background**

The yeast 4830 background (MATa kar1-1 ade2-1 SUQ5 trp1 lys1 leu2 ura3 Δsup35: kanMX [pin−][psi−][ρ−]) was a kind gift from Dr. R. B. Wickner. To be viable, it carries one of the following plasmids:

1. YCp33-I-SUPF: CEN SUP35 (reference sequence) URA3 SUP35 promoter (ref. 11)
2. pDB101: CEN SUP35 (reference sequence) LEU2 SUP35 promoter (refs. 37,45)
3. pDB89: CEN SUP35(E9 sequence) LEU2 CUP1 promoter (refs. 37,45)
4. YCp111-I-SUPF(Q6P): CEN SUP35 (reference sequence with Q6P mutation) LEU2 SUP35 promoter (this study)
5. YCp111-I-SUPF(G43P): CEN SUP35 (reference sequence with G43P mutation) LEU2 SUP35 promoter (this study)
6. YEpl81-I-SUPF(E9): 2µ SUP35(E9 sequence) LEU2 SUP35 promoter (this study)

Plasmids (1) to (6) were transformed to 2 independent stocks of 4830 carrying Plasmid (0). Transformants were selected on SC-Leu plates, and then replicated onto 5-FOA plates to counter-select colonies that had lost Plasmid (0). The Sup35 coding sequences of the Leu+, ura− colonies were reconfirmed.

In contrast to Plasmid (6), whose propagation in the 4830 [psi−] background resulted in red colonies, cells carrying Plasmid (3) had light-pink colonies, indicating mild nonsense suppression due to low Sup35 expression. While the VH, VK, and VL strain were cytoduced without impediment into 4830 carrying Plasmid (6) (51VH/51, 50VK/50, and 56VL/56), there was no prion transmission to 4830 with Plasmid (3).

**Cytoduction**

Cytoduction is a disrupted mating where 2 cells fuse but the nuclei do not. Subsequent cell divisions cause the offspring to inherit one parental nucleus but a mixed cytoplasm. The occurrence of cytoduction is promoted if either of the mating partners carries the kar1-1 allele, which makes nuclear fusion less efficient. 49 74-D-694 (URA3) derivatives were crossed with 4830 to select cytoductants that inherited the 4830 nucleus. Cells were mixed on YPD plates at 30°C for 6 hours, and then streaked on SG-Leu. His plates (containing glycerol as the sole carbon source and lacking leucine and histidine) to select cytoductants that inherited the chromosomal HIS3 and plasmid LEU2 markers of 4830 nuclei and the healthy mitochondria ([ρ−]) from 74-D-694. Diploid colonies could
also pass the selection, but the robust $ADE1^+$ $ADE2^+$ genotype and other heterotic interactions gave them the large, white appearance that was conspicuously different. To be sure, in all experiments, colonies on SG-Leu, His plates were transferred to fresh plates, incubated at 30°C for 36 hours, and then replicated to 5-FOA plates to counter-select cytoductants (i.e. to kill $URA3$ diploids), whose mating type were further confirmed to be $MATa$ (as 4830). For cytoduction in the reverse direction, $[PSI^+]$ $[\rho^+]$ 4830 background (cytoplasm donor) was mixed with a 74-D-694 ($URA3$) derivative ($MATa$ $his3^-$ $[\rho^+]$ $[pin^-][psi^-]$; cytoplasm recipient). Cytoductants were selected on SG-Ura, Lys plates and confirmed to be histidine auxotroph and of the $MATa$ mating type.

**Plasmid Shuffle**

Plasmid shuffle was performed according to Chen et al. with important modifications.19,20

(1) Forward shuffle. Two independently obtained 74-D-694 derivative ($\Delta SUP35$ $[YCp33-I-SUPF(Ura3)][VK]$ $[pin^-]$) was transformed with the multi-copy plasmid YEp181-I-SUPF ($2\mu$ $LEU2$ $SUP35$ promoter) and its mutant versions (e.g., YEp181-I-SUPF(N21P), YEp181-I-SUPF(Y35P)...etc). (To delete $SUP35$, the chromosomal copy was first replaced with the $loxP$: $kanMX$: $loxP$ cassette, which was then excised by transient expression of Cre recombinase.50) Ten to 20 transformants, selected on SC-Ura, Leu plates, were randomly chosen to arrange on YPD plates, which were then incubated at 30°C overnight and subsequently replicated to 5-FOA and YPD plates (Plates I). After 48-hour incubation at 30°C, the 5-FOA plates were replicated to fresh YPD plates again (Plates II). We estimated transmission barriers qualitatively by counting red sectors on Plates II and comparing them with corresponding colonies on Plates I.

(2) Marker switch. At least 4 $[PSI^+]$ colonies for each mutant were selected from Plate II. For complete coverage, colonies exhibiting all shades of pink color were selected if they appeared. They were transformed with a multi-copy plasmid carrying a $URA3$ marker and expressing the same mutant Sup35 from the native promoter (YEpl95-I-SUPF’s). Several patches of the transformants, selected on SC-URA plates, were grown in 3 ml SC-URA liquid media at 30°C for 72 hours in a shaker to lose the YEpl81-I-SUPF ($LEU2$) plasmids.

(3) Reverse shuffle: The $Ura^+$ $leu^-$ colonies were transformed with the centromere-based plasmid YCp111-kanMX-I-SF, carrying a $LEU2$ marker, the kanMX dominant marker (G418 resistant), and the wild type $SUP35$ with the cognate promoter. Six to 12 transformants, randomly selected from SC-Ura, Leu plates, were arranged on YPD plates, grown at 30°C overnight, and replicated to 5-FOA and YPD plates (Plates III). After 48-hour incubation at 30°C, the 5-FOA plates were replicated to fresh YPD plates again (Plates IV). Reverse transmission barriers were estimated by comparing Plates VI with Plates III.

(4) Strain typing: $[PSI^+]$ colonies/sectors were selected from Plates IV under a dissection microscope, and transferred to YPD plates. Strain typing was performed by mating with cells of 74-D-694 ($[pin^-][psi^-]$), transformed with the 2 sets of strain typing plasmids described above (see $[PSI]$ strain typing). A hygromycin-resistant dominant marker was inserted into the plasmids to allow selection of resultant diploids on YPD plates containing 200 $\mu$g/ml G418 and 200 $\mu$g/ml hygromycin B. 74-D-694 cells develop more vigorous colony color on YPD plates than SC, hence the set-up. The Sup35 (1-134) sequences of selected colonies and their parents derived at Step (2) were reconfirmed.
DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ACKNOWLEDGMENTS

We thank Dr. R. Wickner and Ms. C.-I Yu for discussion; Drs. D. Bateman, R. Wickner, and D. Liu for yeast strains and plasmids; Ms. C.-I Yu for unpublished material; Ms. S.-P. Lee, Ms. S.-P. Tsai, Ms. W.-L. Pong at IMB Image Core Facility, Ms. H. J. Huang at Cryo-EM Core Facility, Academia Sinica and Dr. J. Wall at STEM Facility, Brookhaven National Laboratory for assistance in microscopy; Ms. Y. Chen and Mr. H.-C. Lee for technical assistance.

FUNDING

This work was supported by Grant 103-2311-B-001-032 from Ministry of Science and Technology, Taiwan and Career development Grant 99-CDA-L07 from Academia Sinica.

SUPPLEMENTAL MATERIAL

Supplemental data for this article can be accessed on the publisher’s website.

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