72. A. Vortkamp *et al.*, data not shown.
73. Our model suggests that PTHrP represses chondrocyte differentiation, acting on prehypertrophic cells just before the onset of Ihh expression. However, the inhibitory PTHrP signal may also act, to some extent, on the proliferating chondrocytes before the prehypertrophic stage. In the mouse, both antibodies to the receptor and PTH-binding studies suggest that a very low level of PTH/PTHrP receptor may be present on the surface of proliferating cartilage cells even though the levels of PTH/PTHrP receptor mRNA are too low to be detected (*72*).
74. We thank R. L. Johnson for the genomic Ihh probes and sequence information; V. Marigo for the probes for Gli and Ptc; B. R. Olsen for the Col-IX and Col-X probes; B. Houston for the Bmp-6 probes; and T. Woolf, L. Wang, and Ontogeny for providing Sonic hedgehog protein, prepared from an unpublished clone provided by H. Roelink. We are grateful to V. Marigo, J. Capdevilla, E. Laufer, and M. Belliveau for critical discussion of the manuscript and to the members of the Tabin lab for technical advice. A.V. was supported by a fellowship of the Human Frontiers Science Program (HFSP) (LT246/94). Supported by a grant from the HFSP (to C.J.T.) and by National Institutes of Health grants DK47038 (to H.M.K.) and DK4723 to (G.V.S.).

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Support for the Prion Hypothesis for Inheritance of a Phenotypic Trait in Yeast

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A cytoplasmically inherited genetic element in yeast, [*PSI*$^+$], was confirmed to be a prionlike aggregate of the cellular protein Sup35 by differential centrifugation analysis and microscopic localization of a Sup35–green fluorescent protein fusion. Aggregation depended on the intracellular concentration and functional state of the chaperone protein Hsp104 in the same manner as did [*PSI*$^+$] inheritance. The amino-terminal and carboxy-terminal domains of Sup35 contributed to the unusual behavior of [*PSI*$^+$]. [*PSI*$^+$] altered the conformational state of newly synthesized prion proteins, inducing them to aggregate as well, thus fulfilling a major tenet of the prion hypothesis.

Mammalian prions cause devastating neurodegenerative disorders (*1*). Unlike conventional pathogens, they are thought to consist entirely of protein—specifically, a normal nuclear-encoded protein, PrP^C, with an altered "scrapie" conformation, PrP^{Sc} (*1*). The key to prion pathology is thought to be the ability of PrP^{Sc} to induce new PrP^C molecules to adopt the altered structure, producing a protein-conformation cascade that causes the disease and gives rise to new infectious PrP^{Sc}.

A similar explanation can account for the otherwise baffling behavior of two genetic factors in yeast, [*PSI*$^+$] and [*URE3*] (*2*). The [*PSI*$^+$] factor increases translational read-through of all three nonsense codons, and is monitored in the laboratory by omnipotent suppression of nonsense mutations (*3*). Although unlinked to any known nucleic acid, [*PSI*$^+$] behaves as a dominant, cytoplasmically inherited genetic element. It bears an unusual relation to the nuclear-encoded protein Sup35 that is reminiscent of the relation between mammalian prions and nuclear-encoded PrP^C (*1–4*).

Normally, Sup35 is a subunit of the translation-release factor that causes ribosomes to terminate translation at nonsense codons. Release activity maps to the COOH-terminal domain (*5*), which is essential for growth (*6*). Sup35's NH_2-terminal domain is not essential and is required only for the propagation of [*PSI*$^+$] (*6*). Mutations in Sup35 can also cause omnipotent nonsense suppression, but unlike [*PSI*$^+$], the mutant phenotypes exhibit Mendelian inheritance (*3*). Remarkably, transient overexpression of Sup35, or just its NH_2-terminal domain, can induce de novo heritable [*PSI*$^+$] elements (*2*, *6*). Moreover, transient overexpression of the chaperone Hsp104 can restore translational fidelity, heritably converting cells from [*PSI*$^+$] to [*psi*$^-$] (*4*).

These observations argue that [*PSI*$^+$] represents the inheritance of a self-perpetuating alteration in the conformation of Sup35, which is initiated by the NH_2-terminal domain and impairs the ability of the COOH-terminal domain to function in translation. Although this mechanism successfully explains many perplexing genetic observations (*2*, *3*), such a revolutionary model for the inheritance of a phenotypic trait demands the support of direct physical evidence, which we provide here.

Insolubility of Sup35 in [*PSI*$^+$] cells. Isogenic [*psi*$^-$] and [*PSI*$^+$] strains of two different genetic backgrounds (*7*) contained the same quantity of Sup35 and Sup45 (Fig. 1A), the other subunit of the translation-release factor (*8*). Thus, the read-through of nonsense codons in [*PSI*$^+$] cells was not due to reduced accumulation of the termination-factor subunits. Nor was it likely due to posttranslational modification. The migration of the Sup35 and Sup45 proteins from [*PSI*$^+$] cells on high resolution two-dimensional gels was identical to that of the proteins from [*psi*$^-$] cells (*9*).

In contrast, the solubility of Sup35 was very different in [*PSI*$^+$] and [*psi*$^-$] cells. Most Sup35 protein in [*PSI*$^+$] lysates pelleted after centrifugation at 12,000*g*; most remained in the supernatant of [*psi*$^-$] lysates. In [*psi*$^-$] lysates, a substantial fraction of Sup35 remained soluble after centrifugation at 100,000*g*; none remained soluble in [*PSI*$^+$] lysates (Fig. 1B). Similar differences in the solubility of Sup35 in [*PSI*$^+$] and [*psi*$^-$] cells were obtained in the early, mid-, and late log phases of growth as well as in cells in the stationary phase (Fig. 1C) (*10*). No difference in the sedimentation properties of total proteins was detected by Coomassie blue staining, nor did immunostaining show any difference in the sedimentation of Sup45, ribosomal protein L3, or the chaperone proteins Hsp70, Hsp90, and Hsp104 (Fig. 1, B and C) (*10*). High salt (1 M KCl), EDTA (50 mM), and ribonuclease A (400 μg/ml) treatments did not reduce the quantity of Sup35 found in the pellet of [*PSI*$^+$] cells, nor did treatments with nonionic detergent (1% Triton X-100) (*10*). Moreover, like PrP^{Sc} (*1*), the Sup35 protein found in these aggregates was resistant to proteolysis (*11*).

Role of the chaperone Hsp104 in Sup35 aggregation. Overexpression of Hsp104, a protein that promotes the resolubilization and reactivation of heat-damaged proteins (*12*), converts cells from [*PSI*$^+$] to [*psi*$^-$] (*6*). If aggregates of Sup35 reflect the presence of [*PSI*$^+$], Sup35 should return to the soluble state after this conversion. When cells were transformed with a centromeric vector expressing Hsp104 from its own promoter, this was indeed the case (Fig. 1D). [In this and all experiments reported here, the [*PSI*$^+$] and [*psi*$^-$] states were confirmed by plating assays on selective media (Fig. 2) (*13*).] A stronger test of the relation between Sup35 aggregates and [*PSI*$^+$] derives from the ability of transient Hsp104 overexpression to heritably cure

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cells of [*PSI*$^+$] (6). A galactose-inducible single-copy vector, which provides uniform expression in all cells, converted more than 80% of [*PSI*$^+$] cells to [*psi*$^-$] after 3.5 hours of galactose induction. In converted cells plated to glucose media without continued selection for the Hsp104 plasmid (*13*), Sup35 was found in the soluble fraction, even though Hsp104 was no longer overexpressed (*10*). Similar results were obtained with a glucocorticoid-inducible Hsp104 expression vector (*10*).

Paradoxically, [*PSI*$^+$] cells also convert to [*psi*$^-$] when Hsp104 expression is eliminated by deletion of the *HSP104* gene (6). This is surprising because the only previously known function of Hsp104 was to promote the dissolution of aggregates of heat-damaged proteins; these aggregates are maintained in cells with *HSP104* deletions (*12*). When [*PSI*$^+$] cells were converted to [*psi*$^-$] through deletion of *HSP104*, Sup35 was found in the soluble fraction (Fig. 1E). Thus, the behavior of Sup35 aggregates paralleled the behavior of [*PSI*$^+$] and differed from the behavior of heat-damaged aggregates. Together these data strongly support the hypothesis that [*PSI*$^+$]-mediated nonsense suppression is due to a conformational alteration in Sup35 that is self-sustaining as long as Hsp104 is present at its normal concentration.

The sedimentation properties of Sup35 also provide a simple molecular explanation for one of the most perplexing aspects of [*PSI*$^+$] biology—the ability of the element to exist in a cryptic form. For example, when [*PSI*$^+$] cells were transformed with plasmids encoding mutations in the Hsp104 nucleotide-binding domains (NBDs) [either K218T (a Lys to Thr substitution at amino acid 218 in NBD 1) or K620T (an analogous substitution in NBD 2)], the [*PSI*$^+$] phenotype was suppressed but not cured (*4*) (Fig. 2B). That is, the cells did not exhibit nonsense suppression and were unable to grow on selective media, but when the plasmid encoding the mutant Hsp104 protein was lost, [*PSI*$^+$] reappeared and growth on selective media was restored. In contrast, the double mutant K218TK620T cured cells of [*PSI*$^+$]; when the expression plasmid was lost, [*PSI*$^+$]-mediated nonsense suppression was not regained (Fig. 2B).

As shown in Fig. 1F, a greater fraction of Sup35 remained soluble in cells expressing K218T or K620T than in the original [*PSI*$^+$] strain, but most of the the protein remained insoluble (Fig. 1F). Presumably, the increase in soluble Sup35 allowed faithful termination at nonsense codons, but a sufficient quantity of aggregated Sup35 remained to reestablish [*PSI*$^+$] when the plasmid encoding the mutant protein was lost. A smaller fraction of Sup35 was insoluble in the double mutant, and this material was unable to act as a prion-inducing element.

Visualizing prion elements. To monitor [*PSI*$^+$] elements in real time in living cells, we used a green fluorescent protein (GFP) fusion (*14*). The NH_2-terminal prion-deter-

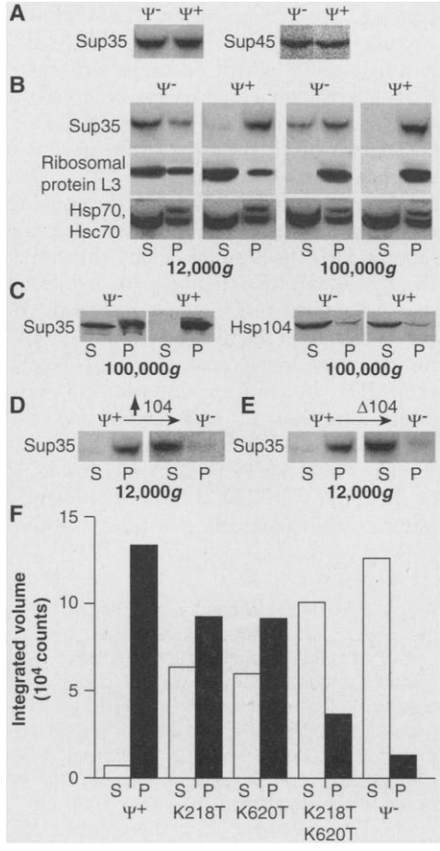

Fig. 1. Sup35 aggregates in [*PSI*$^+$] but not [*psi*$^-$] cells. (**A**) Sup35 and Sup45 accumulation in [*PSI*$^+$] and [*psi*$^-$] cells. Electrophoretically separated total cellular proteins were reacted with antibodies directed against Sup35 and Sup45 (*7, 22, 25*). (**B**) Solubility of Sup35 in mid–log phase cells. Proteins fractionated by centrifugation at 12,000 or 100,000*g* were electrophoretically separated and reacted with antibodies against Sup35 (*22*), ribosomal protein L3 (*25*), and members of the Hsp70 and Hsc70 family [monoclonal antibody (mAb) 7.10]. (**C**) Solubility of Sup35 in stationary-phase cells. Proteins were analyzed as in (B) and reacted with antibodies to Sup35 (*22*) and Hsp104 (*12*). Hsp104 is more readily visualized in stationary-phase cells because Hsp104 concentrations are higher, but similar results were obtained in log-phase cells (*10*). (**D**) Sup35 is soluble in cells overexpressing Hsp104. [*PSI*$^+$] 74D-694 cells (Ψ^+) were converted to [*psi*$^-$] (Ψ^-) by transformation with a centromeric plasmid pYS104 carrying the wild-type *HSP104* gene, which increases Hsp104 expression two- to threefold as compared with that of wild-type cells (*4*). Fractionated lysates were analyzed as in (B). (**E**) Sup35 is soluble in *HSP104* deletion mutants (Δ104). [*PSI*$^+$] 74D-694 cells (Ψ^+) were converted to [*psi*$^-$] (Ψ^-) by transformation with a construct [*hsp104::Leu2*] (*4*) that abolishes Hsp104 expression by disrupting the chromosomal *HSP104* gene. Fractionated lysates were analyzed as in (B). (**F**) Solubility of Sup35 in cells harboring cryptic [*PSI*$^+$] elements. [*PSI*$^+$] 74-D694 cells were converted to [*psi*$^-$] by transformation with centromeric plasmids encoding Hsp104 proteins with Lys to Thr substitutions in the first (K218T) or second (K620T) or both (K218TK620T) NBDs (*4*). The parental strain (Ψ^+) and its isogenic [*psi*$^-$] derivative (Ψ^-) are also shown. Immmunoblots were quantified with ImageQuant software (Molecular Dynamics).

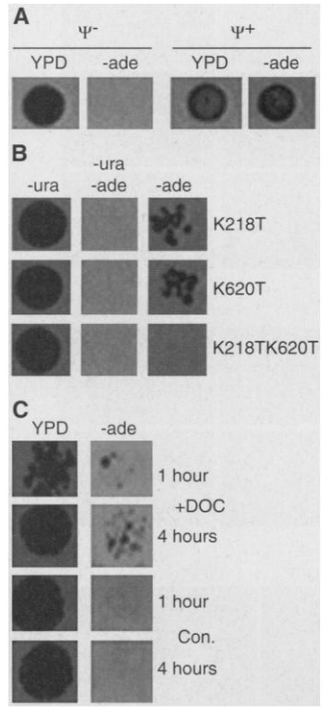

Fig. 2. Analysis of [*PSI*$^+$] propagation by colony formation. (**A**) Read-through of nonsense codons in [*PSI*$^+$] cells detected by the suppression of nonsense mutations. In 74-D694 cells, the suppressible marker is *ade 1-14* (UGA). [*psi*$^-$] cells (Ψ^-) do not form colonies on adenine-deficient medium (–ade) and are red on YPD; [*PSI*$^+$] cells (Ψ^+) form colonies on adenine-deficient medium and are white on YPD (*4, 13*). (**B**) Cells expressing K218T or K620T Hsp104 mutations carry cryptic [*PSI*$^+$] elements. [*PSI*$^+$] cells analyzed in Fig. 1F were spotted onto plates deficient in uracil (–ura) or adenine (–ade) or both. Growth without uracil forces retention of the plasmid. Growth on adenine-deficient medium requires read-through of the *ade 1-14* UGA codon and the reappearance of [*PSI*$^+$]; it occurs only when cells are allowed to lose the K218T or K602T expression plasmid. (**C**) Transient expression of NPD-GFP induces [*PSI*$^+$]. [*psi*$^-$] 74-D694 cells carrying the *GRE*-regulated NPD-GFP expression plasmid (*15*) were treated with DOC (1 μM) for 1 or 4 hours. Equal numbers of induced (+DOC) and uninduced control (con.) cells were spotted onto YPD and adenine-deficient medium (–ade). [*psi*$^-$] strains underwent conversion to [*PSI*$^+$] after as little as 1 hour of induction, and the extent of conversion increased over time. NPD-GFP did not influence colony formation by [*PSI*$^+$] 74-D694 cells (*16*).

mining domain (NPD) of Sup35 was fused to GFP and placed under the control of the regulatable promoters *CUP1* (inducible with copper) and *GRE* [inducible with 11-deoxycorticosterone (DOC)] (*15*). When NPD-GFP was induced by either copper or DOC, fluorescence was diffusely distributed in two different [*psi*$^-$] strains (*7*). In their isogenic [*PSI*$^+$] derivatives, as soon as fluorescence could be detected it was concentrated in a small number of intense foci (Fig. 3A). When subjected to differential centrifugation, NPD-GFP sedimented with Sup35 in [*PSI*$^+$] lysates but remained in the supernatant of [*psi*$^-$] lysates (*10*). When expressed without the NPD, GFP was diffusely distributed and soluble in both [*psi*$^-$] and [*PSI*$^+$] cells (Fig. 3B). Thus, the coalescence of GFP in [*PSI*$^+$] strains depended on both the attached NPD and the presence of preexisting [*PSI*$^+$] elements.

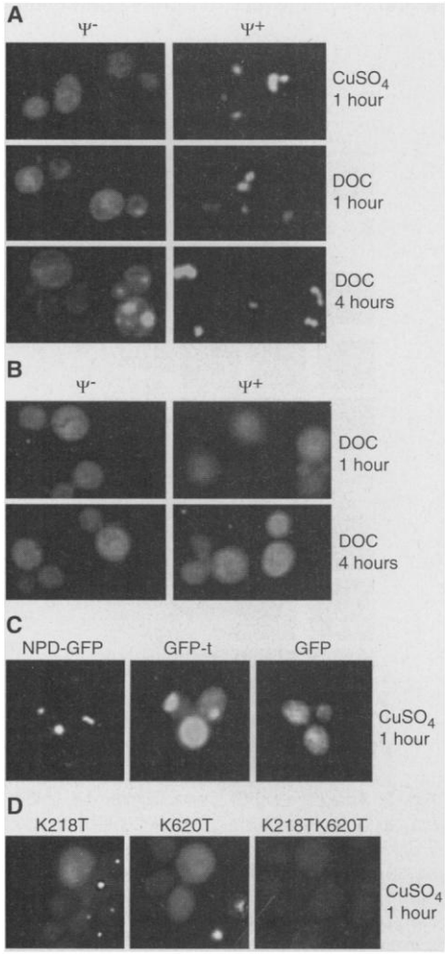

Fig. 3. Visualization of protein aggregates with GFP. (**A**) Diffuse distribution of NPD-GFP in [*psi*$^-$] strains (Ψ$^-$) and coalescence in [*PSI*$^+$] strains (Ψ$^+$). Isogenic [*psi*$^-$] and [*PSI*$^+$] cells (74D-694) were transformed with a *CUP1* or *GRE* NPD-GFP expression plasmid (*15*). $CuSO_4$ (50 μM) or DOC (1 μM) was added to log-phase cultures for 1 or 4 hours. Because our antibodies do not recognize Sup35 protein in its native state, we could not perform colocalization studies. (**B**) GFP is diffusely distributed in all cells. Analysis was as in (A), except that the *GRE* regulated plasmid encoded GFP without the NPD domain. (**C**) The heritability of GFP fluorescence patterns in groups of budding cells. Analysis was as in (A), with plasmids encoding NPD-GFP, GFP-t, and GFP (*15*) under the control of a *CUP1* promoter. (**D**) Cryptic [*PSI*$^+$] elements visualized by NPD-GFP fluorescence. Analysis was as in (A), (*CUP1*-regulated NPD-GFP) in cells transformed with plasmids expressing mutant Hsp104 proteins (see Fig. 1F).

The Sup35 NPD can induce [*PSI*$^+$] elements in [*psi*$^-$] cells (*6*). In our study, aggregates appeared in a small percentage of the copper-treated [*psi*$^-$] cells after 1 hour of induction (*16*). When plated onto media selective for nonsense suppression but not selective for the NPD-GFP plasmid, heritable [*PSI*$^+$] elements were detected in a similar small percentage of cells (*16*). When the NPD-GFP fusion protein was expressed in [*psi*$^-$] cells at a higher level or for a longer period, bright points of coalescence appeared in a larger fraction of the cells (Fig. 3A) (*16*), and a correspondingly larger fraction showed conversion to [*PSI*$^+$] (Fig. 2C). Intense fluorescent foci were maintained in mother and daughter cells for at least 4 hours after NPD-GFP expression was repressed. In contrast, when NPD-GFP was expressed at high levels in an *HSP104* deletion strain, which cannot propagate [*PSI*$^+$], GFP coalescence was observed in only a few rare cells (*16*). Thus, NPD-GFP coalescence is a marker of the heritable prionlike state of Sup35.

Next we used NPD-GFP to visualize cryptic [*PSI*$^+$] elements in cells expressing mutant Hsp104 proteins. Cells expressing the K218T and K620T proteins exhibited the diffuse fluorescence characteristic of [*psi*$^-$] cells, but many also contained the intense foci characteristic of [*PSI*$^+$] cells (Fig. 3D). In contrast, intense foci were rarely observed in cells expressing the K218TK620T double mutant. Thus, although some Sup35 protein was insoluble in the latter (Fig. 1F), it did not efficiently nucleate the coalescence of newly synthesized NPD-GFP nor the reappearance of [*PSI*$^+$] (Fig. 2B).

Unique properties of Sup35 [*PSI*$^+$] aggregates. To further probe the relation between protein aggregation and prion inheritance, we monitored the behavior of another aggregation-prone GFP protein, a run-on translation product generated by mutation of the termination codon (*15*). This protein (GFP-t) was more variable in expression than NPD-GFP, accumulating in only a fraction of the cells. In these, its distribution varied widely: In groups of budding cells, some individuals exhibited diffuse fluorescence, whereas others showed intense concentrated foci (Fig 3C). Thus, unlike that of NPD-GFP, the distribution pattern of GFP-t was not inherited.

During the formation of aggregates, other amyloids and prions are thought to abandon most of their normal structure. Because GFP fluorescence depends on proper tertiary structure (*17*), some structure must be maintained in the prionlike foci of NPD-GFP. We do not yet know whether the COOH-terminal domain of Sup35 retains its tertiary structure during prion formation, but further experiments demonstrated that it strongly affected the behavior of the NPD.

To determine how Hsp104 affects the solubility of the NH_2- and COOH-terminal domains of Sup35, we expressed them separately in wild-type cells and in *HSP104* deletion mutants (Fig. 4). In wild-type cells, each domain was present in the supernatant and pellet fractions after a 100,000*g* spin. In *hsp104* deletion mutants, the distribution of the COOH-terminal domain was unchanged, but the NH_2-terminal domain was found only in the pellet. Apparently, the NH_2-terminal domain has an intrinsic ability to interact with Hsp104, and through this interaction to undergo a change in state that alters its solubility (*18*). The aggregates formed by the NPD alone, however, behaved like the amorphous aggregates of denatured proteins that accumulate after heat shock and remain insoluble in *HSP104* deletion mutants (*12*). This contrasted with the be-

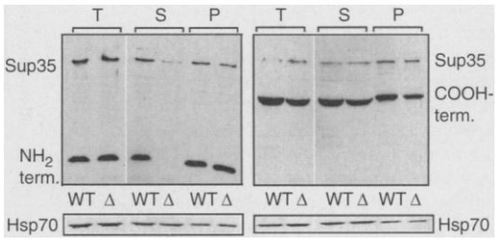

Fig. 4. Hsp104 influences the solubility of the Sup35 NH_2-terminal domain. [*psi*$^-$] cells (74-D694) with an intact (WT) or disrupted (Δ) chromosomal *HSP104* gene transformed with high-copy-number plasmids (*6*) encoding either the Sup35 NH_2-terminal (NH_2-term.) or COOH-terminal (COOH-term.) domain. Lysates were subjected to centrifugation at 100,000*g*. Total lysate (T), supernatant (S), and pellet (P) were analyzed as in Fig. 1, with a polyclonal antiserum to Sup35 and mAb 7.10. Immune complexes were visualized with horseradish peroxidase–conjugated protein A and ECL reagent (Amersham).

havior of the NPD in its normal context, attached to the COOH-terminus, where Hsp104 was actually required for aggregation (Fig. 1D) and, moreover, was required at an intermediate concentration. Thus, the COOH-terminal domain of Sup35 profoundly alters the properties of the NPD and the consequences of its interactions with Hsp104.

The prion hypothesis in yeast. Our data demonstrate that Sup35 undergoes a change in state when cells convert from [*PSI*$^+$] to [*psi*$^-$] and from [*psi*$^-$] to [*PSI*$^+$]. This change involves the disappearance and appearance of a unique heritable aggregate that rapidly captures newly synthesized proteins containing the Sup35 NPD and is governed by the chaperone Hsp104 (Fig. 5). The ability of preexisting [*PSI*$^+$] elements to alter the conformational fate of newly synthesized prion proteins provides direct physical support for the prion hypothesis of [*PSI*$^+$] inheritance (*2*).

Aggregation is a hallmark of the change in state associated with the conversion of mammalian PrP^C to PrP^{Sc} (*19*). The many correlations we observed between the insolubility of Sup35 and the presence of [*PSI*$^+$] demonstrate that aggregation is characteristic of yeast prions as well. However, three findings indicate that [*PSI*$^+$] is more than a simple consequence of protein aggregation. First, a substantial fraction of Sup35 remained insoluble in cells expressing the K218TK620T double mutant, yet this material did not efficiently seed the propagation of [*PSI*$^+$] nor the coalescence of NPD-GFP. Second, when GFP was induced to aggregate through a COOH-terminal extension, the aggregates it formed were not heritable. Third, the aggregates formed by the NPD of Sup35 alone were affected by an *Hsp104* deletion in a different manner than were the [*PSI*$^+$] aggregates of wild-type Sup35. We suggest that the COOH-terminal domain affects the packing of the Sup35 aggregates in a manner that is essential to [*PSI*$^+$] propagation.

Genetic analysis of yeast prionlike elements and the application of GFP fusion protein technology provide a supplement to mammalian investigations that should speed our understanding of self-propagating changes in protein structure and may lead to new approaches for therapeutic intervention in neurodegenerative diseases. But this work, together with work on another such yeast element, [*URE3*] (*2*, *20*), has yet broader implications. The existence of prions—elements of inheritance arising from alternative protein conformations—in both mammals and yeast suggests that they are broadly distributed in nature. In the mammalian brain, a nonmitotic tissue, prions were revealed by their capacity to function as infectious agents; in yeast, they were revealed by their ability to produce heritable changes in phenotype. A wide variety of elusive epigenetic phenomena in other organisms may well prove to depend on the maintenance of alternative protein structures. Finally, because the inheritance of the yeast [*PSI*$^+$] elements depends on Hsp104, a chaperone induced by environmental stress, this phenomenon provides a plausible mechanism for the inheritance of an environmentally acquired characteristic.

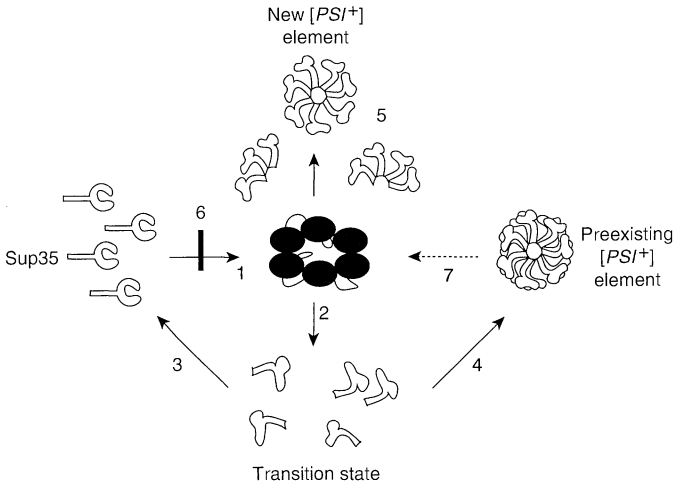

Fig. 5. A model for prion formation in yeast. 1: Newly synthesized Sup35 (white shapes at left) interacts with the chaperone Hsp104 (black ovals at center). 2: Hsp104 helps Sup35 achieve a protein-folding transition state that is required for prion formation but is inherently unstable. 3: In the absence of [*PSI*$^+$], Sup35 reverts to its normal functional state. 4: Preexisting [*PSI*$^+$] elements capture and stabilize transition-state conformers; Sup35 is sequestered from translation and unfaithful termination leads to nonsense suppression. 5: Transient overexpression of Sup35 nucleates prions de novo because the high concentration of transition-state conformers increases the likelihood of stabilizing intermolecular interactions (*23*). 6: In the absence of Hsp104, the transition state is difficult to attain and prions cannot be perpetuated. 7: Overexpression of Hsp104 might disturb the equilibrium in several ways: Hsp104 might bind prion-state conformers and disaggregate them; rebind monomers, reducing their ability to be captured by [*PSI*$^+$] elements; or reduce the local concentration of transition-state conformers because they are dispersed in association with larger numbers of Hsp104 (*24*).

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7. Shown in Fig. 1A is *Saccharomyces cerevisiae* strain 74D-694 (*MAT**a**, ade1 his3 leu2 trp1 ura3*) [*PSI*$^+$] and [*psi*$^-$] derivatives. Not shown is strain D1142-1A (*4*).
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9. The first dimension of the gel was performed with the Immobiline Dry Strip system (linear gradient, pH 4 to 7) and the second was performed on an ExcelGel SDS (gradient 8 to 18%) with a Multiphor II apparatus. For cell lysis (of strain 74D-694; [*PSI*$^+$] and [*psi*$^-$]), cells were grown on yeast extract, peptone, and dextrose (YPD) to 5×10^6 cells per milliliter and electrophoresis was done according to the manufacturer's recommendations (Pharmacia Biotech). Total proteins were stained with Coomassie blue, transferred to Immobilon filters, and reacted with antibodies to Sup35 (peptide amino acids 137 to 151). Immune complexes were visualized as described in Fig. 4 (M. M. Patino and S. Lindquist, data not shown).
10. M. Patino and S. Lindquist, data not shown.
11. When cell lysates were treated with proteinase K, the Sup35 protein of [*PSI*$^+$] cells was more resistant to digestion than was that of [*psi*$^-$] cells, but unlike the case with PrP^{Sc}, no specific protease-resistant fragments of Sup35 were detected (M. Patino, S. Lindquist, Y. Chernoff, unpublished data).
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13. In strain 74D-694, the [*PSI*$^+$]^{ts} suppressible marker is *ade 1-14*, a UGA codon. [*PSI*$^+$] cells produce white colonies on complete media (YPD) and grow without adenine supplementation; [*psi*$^-$] cells produce red colonies and require adenine. Cryptic [*PSI*$^+$] elements in 74D-694 cells expressing Hsp104 K218T or K620T proteins from *URA* vectors were detected by simultaneous platings on media (i) with adenine and without uracil (all cells grew), (ii) with neither uracil or adenine (maintenance of the plasmid abrogated [*PSI*$^+$]-mediated nonsense suppression and cells did not grow), and (iii) with uracil and without adenine (the small percentage of cells that had spontaneously lost the plasmid at the time of plating recovered [*PSI*$^+$]-mediated nonsense suppression and grew). Cells expressing the double mutant could not recover [*PSI*$^+$] and did not grow on medium with uracil and without adenine.
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15. To create GFP-t, a DNA segment encoding GFP but lacking the natural termination codon was amplified with primers 5′-CGGATCCCATATGAGTAAAGGAGAAGAACTT-3′ and 5′-CTCGAGCTCACTAGTTTTGTATAGTTCATCATCCATGCC-3′. The polymerase chain reaction (PCR) product was subcloned into plasmid p2UGpd (pRS426) with GPD promoter (D. Nathan and S. Lindquist, unpublished data) as a Bam HI–Sac I fragment generating the plasmid p2UGFP-t. The complete open reading frame extends into the vector sequence and encodes an aggregation-prone GFP with a 46-residue COOH-terminal extension with the sequence TSELQLLFPLVRVNCALGVIMVIAVSCVKLLSAHNSTQHTSRKHKV-COOH (*21*). To create an unfused GFP, a termination codon was regenerated by cutting p2UGFP-t with Spe I, filling in the overhanging ends, and re-ligating (creating the plasmid p2UGFP). To create NPD-GFP, the DNA encoding the NPD of Sup35 was amplified from pEMBL-SUP2 (*6*) with primers 5′-CGCGGATCCATGTCGGATTCAAACC-3′ and 5′-CG-

CGGATCCATCGTTAACAACTTCG-3′ and subcloned as a Bam HI fragment into p2UGFP. The resulting construct encodes the NH_2-terminal 253 residues of Sup35 fused to GFP. Each of these constructs (GFP-t, GFP, and NPD-GFP) was amplified by PCR and subcloned into p2UG [M. Schena, D. Picard, K. R. Yamamoto, *Methods Enzymol.* **194**, 389 (1991)], conferring DOC-inducible expression, and also into pCLUC [D. J. Thiele, *Mol. Cell. Biol.* **8**, 745 (1988)] for copper-inducible expression. Fidelity of constructs was confirmed by dideoxy nucleoside triphosphate sequencing, and the mobility of the expressed proteins was determined by protein immunoblot analysis.
16. J.-J. Liu and S. Lindquist, data not shown.
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18. The ability of the NH_2-terminal domain to stimulate Hsp104 adenosine triphosphatase activity suggests that this interaction is direct (E. Schirmer and S. Lindquist, unpublished data).
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21. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
22. Isogenic [*PSI*$^+$] and [*psi*$^-$] cells were grown to a density of ~5 × 10^6 cells per milliliter in YPD. Cells were suspended in 50 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, cycloheximide (100 μg/ml), 1 mM benzamidine, 2 mM phenylmethylsulfonyl, leupeptin (10 μg/ml), pepstatin A (2 μg/ml), and ribonuclease A (100 μg/ml) and disrupted with glass beads at 4°C. Proteins resolved by SDS–polyacrylamide gel electrophoresis were transferred to Immobilon membranes (Millipore) and reacted with an antiserum against amino acids 137 to 151 of Sup35 or against Sup45 (gift of M. Tuite), followed by ^{125}I-conjugated protein A (ICN Pharmaceuticals), and then exposed to a PhosphorImager screen (Molecular Dynamics).
23. These interactions may be facilitated by the simultaneous binding of several Sup35 proteins to an Hsp104 hexamer or by rapid sequential binding and release of individual conformers in its immediate vicinity. [*PSI*$^+$] is drawn as an ordered aggregate, with reference to a model for mammalian prion formation (*19*). [*PSI*$^+$] aggregates have special properties, but we do not yet know if they form an ordered structure.
24. In preliminary experiments, GFP-marked prions do not disaggregate rapidly when Hsp104 is overexpressed (J.-J. Liu, unpublished data). Given their size, this is not surprising. However, because the prion assay relies on colony formation, and because Hsp104 is long-lived, it is possible that overexpression of Hsp104 simply prevents new prion conformers from joining the prion while preexisting prions are diluted by cell division.
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