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Antiprion systems in yeast cooperate to cure or prevent the generation of nearly all [*PSI*$^+$] and [URE3] prions

Moonil Son[a,1] and Reed B. Wickner[a,2]

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[*PSI*$^+$] and [URE3] are prions of *Saccharomyces cerevisiae* based on amyloids of Sup35p and Ure2p, respectively. In normal cells, antiprion systems block prion formation, cure many prions that arise, prevent infection by prions, and prevent toxicity of those prions that escape the other systems. The *upf1Δ*, *ssz1Δ*, and *hsp104*^{T160M} single mutants each develop [*PSI*$^+$] at 10- to 15-fold, but the triple mutant spontaneously generates [*PSI*$^+$] at up to ∼5,000-fold the wild-type rate. Most such [*PSI*$^+$] variants are cured by restoration of any one of the three defective antiprion systems, defining a previously unknown type of [*PSI*$^+$] variant and proving that these three antiprion systems act independently. Generation of [*PSI*$^+$] variants stable in wild-type cells is also increased in *upf1Δ ssz1Δ hsp104*^{T160M} strains 25- to 500-fold. Btn2 and Cur1 each cure 90% of [URE3] prions generated in their absence, but we find that *btn2Δ* or *cur1Δ* diminishes the frequency of [*PSI*$^+$] generation in an otherwise wild-type strain. Most [*PSI*$^+$] isolates in a wild-type strain are destabilized on transfer to a *btn2Δ* or *cur1Δ* host. Single *upf1Δ* or *hsp104*^{T160M} mutants show the effects of *btn2Δ* or *cur1Δ* but not *upf1Δ ssz1Δ hsp104*^{T160M} or *ssz1Δ hsp104*^{T160M} strains. The disparate action of Btn2 on [URE3] and [*PSI*$^+$] may be a result of [*PSI*$^+$]'s generally higher seed number and lower amyloid structural stability compared with [URE3]. Thus, prion generation is not a rare event, but the escape of a nascent prion from the surveillance by the antiprion systems is indeed rare.

prion | antiprion system | [*PSI*$^+$]

Significance

The yeast infectious proteins (prions) [*PSI*$^+$] and [URE3] are filamentous polymers (amyloids) of Sup35p and Ure2p. Antiprion systems cure most prions as they arise, inhibit prion generation, limit prion pathogenesis, and block prion infection. We find that mutants lacking several antiprion systems develop [*PSI*$^+$] prions at up to 5,000-fold the frequency of a normal strain, including many prion variants cured by any of the several antiprion systems. Btn2 and Cur1 cure most [URE3] variants but promote [*PSI*$^+$] generation and propagation. Btn2 is known to sequester [URE3] amyloid (of Ure2p) and other misfolded proteins, possibly explaining Btn2's distinct action on [URE3] and [*PSI*$^+$]. Understanding yeast antiprion systems may facilitate discovery of analogous/homologous human systems useful in dealing with amyloid diseases.

The yeast prions [*PSI*$^+$] and [URE3] are infectious amyloidoses of Sup35p and Ure2p, respectively (refs. 1–4; reviewed in refs. 5 and 6). The normal translation termination effect of Sup35p (7, 8) and the nitrogen catabolite repression effect of Ure2p (9) are impaired in the respective prion-containing strains (1). The resulting translation read through of stop codons or transcription derepression of *DAL5* has been engineered to produce a convenient Ade+ (white) or Ura+ phenotype for prion-containing cells and Ade− (red) or Ura− for normal cells (10–12).

Amyloid is a linear polymer of a single protein, but a given prion protein can form many amyloid "variants," differing in their biological and structural properties (e.g., refs. 13–15). Each variant amyloid templates its structure to previously unknown molecules, joining the ends of the filaments by a mechanism suggested by its in-register parallel folded β-sheet architecture (16, 17). The amyloid filaments of the prion protein are distributed vertically (to offspring) and horizontally (through mating) so that the prion is heritable and infectious. The prion variants may be viewed as alleles of a nonchromosomal gene.

The rather low frequency of [*PSI*$^+$] and [URE3] in wild strains, the high proportion of variants of each of these prions that are lethal or very toxic, and the stress response seen in cells with either prion indicate that these two prions are generally detrimental (reviewed in ref. 6). Supporting this view is the finding that there is an array of antiprion systems blocking infection with a prion, limiting the generation of new prions, curing most of the prions that do arise, and reducing the ill effects of some of those prion variants that get past the other antiprion systems (reviewed in ref. 18).

Normal levels of the paralogous Btn2 and Cur1 cure >90% of the [URE3] variants arising in their absence (19). Btn2 acts by collecting (sequestering) the Ure2p amyloid filaments at one location in the cell so that often, one of the daughter cells receives no amyloid and is cured (20). Cur1 does not colocalize with Ure2p amyloid aggregates as [URE3] is being cured (20, 21), and its mechanism of action is not clear. Btn2 and Cur1 cure an artificial prion, and Btn2 collects other cellular aggregates; therefore, they are not specific for [URE3] (22, 23), but Btn2 and Cur1, even when overproduced, cure almost no [*PSI*$^+$] variants (20, 24). Btn2 and Cur1 curing of [URE3] each requires Hsp42, but neither Btn2 nor Cur1 requires the other (19). Overproduction of Hsp42, which does colocalize with nonprion aggregates (25), also cures the [URE3] prion in a process requiring Cur1 (but not Btn2) (19). Like Btn2, Hsp42 is now

Author affiliations: [a]Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Disease, NIH, Bethesda, MD 20892-0830

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[1]Present address: Department of Microbiology, Pusan National University, Busan 46241, Republic of Korea.

[2]To whom correspondence may be addressed. Email: wickner@helix.nih.gov.

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known to have "sequestrase" activity (26). Btn2 and Cur1 are elevated ~200-fold, much more than any other protein (of >4,600 examined), in mutants interfering with proteasome assembly or lacking one of the proteasome subunits, suggesting that their action on aggregates is a "plan B" when proteasomes are overwhelmed (27). The proteasome inhibitor, MG132, or elevated temperature also results in elevated Btn2p and Cur1p (21, 23). Overproduced Cur1 (but not Btn2) enhances the [*PSI*$^+$] phenotype (21), and overproduced Btn2 or Cur1 also partially sequesters Sis1p (an Hsp40) in the nucleus, an effect proposed to account for their prion curing (21, 23); however, as discussed below, we find this model inconsistent with the data.

The ribosome-associated chaperones Ssb1/2, Zuo1, and Ssz1 are responsible for ensuring that nascent proteins fold properly (28, 29). Mutants lacking both of the closely similar Ssb1 and Ssb2 show elevated frequency of [*PSI*$^+$] (30), and the same is true for *zuo1*Δ or *ssz1*Δ mutants (31, 32). Prion variants arising in these mutants include variants that cannot propagate in a wild-type strain (33). The function of these chaperones in facilitating normal folding of nascent proteins would suggest that any prion might be more frequent in these mutants, but no effect on [URE3] generation was observed (33).

Hsp104 is a protein disaggregase, which with Hsp70s, nucleotide exchange factors, and Hsp40s, extracts single-prion protein molecules from the amyloid filament, thus breaking the filaments and generating new fiber ends (prion replication) (34–37). In addition to this central role in allowing amyloid-based prions to propagate, overproduced Hsp104 cures all [*PSI*$^+$] variants (34) but cures many [*PSI*$^+$] variants at normal levels of the protein (38). Mutants in the N terminus of *HSP104* (e.g., *hsp104*^{T160M}) completely lose the prion-curing activity with little effect on the disaggregase activity, the prion propagation activity, or the protection of cells from heat shock, indicating that this prion-curing activity of Hsp104 is distinguishable from the prion propagation activity (disaggregase) (39). [*PSI*$^+$] arises spontaneously in *hsp104*^{T160M} strains at ~13-fold the wild-type rate, and many of the [*PSI*$^+$] variants arising in the mutant are cured by transfer to a wild-type host (38, 40). The frequency of generation of [*PSI*$^+$] variants that can propagate in the wild type is also elevated (38).

An mRNA with a premature stop codon is generally degraded much faster than the normal mRNA, a phenomenon called nonsense-mediated decay (NMD) that is carried out by the ribosome-associated complex of Upf1, Upf2, and Upf3 (reviewed in ref. 41). The Upf complex is normally associated with Sup35 on the ribosome (42). In *upf* mutants, the frequency of [*PSI*$^+$] generation is elevated >10-fold, an effect not due to the loss of NMD in the mutants but rather, due to the loss of the normal association of Sup35 with the Upf complex (43). In vitro amyloid formation by Sup35 (but not Ure2) is inhibited by submolar concentrations of Upf1 and Upf1 associates with Sup35 amyloid filaments in vivo (43) and in vitro (42). It is suggested either that the Upf complex competes with amyloid filaments for binding to Sup35p monomers or that the Upf complex binds to the ends of filaments, blocking their elongation.

The highly phosphorylated inositol polyphosphates are needed for propagation of most [*PSI*$^+$] variants, and some variants are unstable without the elevated levels of 5-pyrophosphate inositol polyphosphates found in *siw14*Δ cells (44). Siw14 thus acts as an antiprion component, but how inositol polyphosphates affect [*PSI*$^+$] is unknown.

In single-antiprion mutants, the elevated frequency of prion generation (spontaneous or following prion protein overexpression) is largely due to the appearance of "hypersensitive" prion variants that are cured if the mutant gene is restored with normal expression. Since the other antiprion genes in such a strain are intact, they are evidently insufficient to cure the prion variants that are hypersensitive to the one mutant gene. In a multiple mutant, each of the variants hypersensitive to a single-antiprion system would be present, resulting in the sum of the single-mutant frequencies being observed. Alternatively, if these hypersensitive variants each require all of the antiprion systems to be cured or if the antiprion systems are all part of one system, a less than additive result would be obtained. We find surprisingly high frequencies of prion generation in some combinations and surprisingly low frequencies in others. Using the combinations of mutations producing these effects and the properties of the prion variants arising in the various strains, we infer the existence of prion variants with properties different from those previously described. We show that Btn2 and Cur1 affect [*PSI*$^+$] generation in the opposite direction from their effects on [URE3] and that the antiprion systems cooperate in eliminating the overwhelming majority of prions arising in the cell. As in our earlier work, we use the antiprion systems' actions on prions to classify genes and the prions' response to these genes to classify prion variants.

Results

Potentiation of Prion Generation by Multiple Mutations in Antiprion Genes. Single *ssz1*Δ, *upf1*Δ, and *hsp104*^{T160M} mutants are reported to show frequencies of spontaneous [*PSI*$^+$] generation 10 to 15×, 9×, and 13× that of the wild type, respectively (30, 33, 38, 43), while *btn2*Δ *cur1*Δ strains had 5× elevated frequency of [URE3] generation (19). The spontaneous [*PSI*$^+$] generation frequency was measured in a set of strains with all combinations of *ssz1*Δ, *upf1*Δ, *btn2*Δ, *cur1*Δ, and *hsp104*^{T160M} (Table 1). We find it convenient to abbreviate the mutant antiprion genes as *s*, *u*, *b*, *c*, and *h*, respectively, as in Table 1. Single *ssz1*Δ, *upf1*Δ, or *hsp104*^{T160M} mutations elevated [*PSI*$^+$] generation at least as much as previously reported.

A first examination of Table 1 shows several remarkable features. First, in an otherwise wild-type strain, *btn2*Δ, *cur1*Δ, and

Table 1. Strains multiply defective for antiprion components have very high [*PSI*$^+$] spontaneous generation frequency

Mutants	$\Psi^+/10^6$	Mutants	$\Psi^+/10^6$
WT	3.3 ± 3.3	*su*	15 ± 4
b	0*	*bsu*	57 ± 12
c	0*	*csu*	5 ± 2
bc	0.1*	*bcsu*	0
h	53 ± 2	*uh*	128 ± 10
bh	4 ± 1	*buh*	6 ± 2
ch	0	*cuh*	0
bch	0	*bcuh*	4 ± 2
s	42 ± 4	*sh*	534 ± 300
bs	22 ± 9	*bsh*	547 ± 40
cs	4 ± 1	*csh*	493 ± 70
bcs	0	*bcsh*	548 ± 400
u	25 ± 8	*suh*	768 ± 110
bu	1 ± 0.6	*bsuh*	1,576 ± 450
cu	1 ± 0.3	*csuh*	350 ± 40
bcu	2 ± 0.1	*bcsuh*	1,320 ± 1,000

The frequency (mean ± SD) of spontaneous guanidine-curable [*PSI*$^+$] clones was measured in strains carrying all combinations of these five mutations in antiprion genes. *b*, *btn2*Δ; *c*, *cur1*Δ; *h*, *hsp104*^{T160M}; *s*, *ssz1*Δ; *u*, *upf1*Δ.
*Differs from WT with $P < 2 \times 10^{-4}$.

Table 2. Direct effects of mutations on translation termination efficiency

Strain	Genotype	Relative Firefly luciferase/Renillia luciferase	
		Experiment 1	Experiment 2
659	WT [*psi*$^-$]	1.00	1.35
592	*h* [*psi*$^-$]	1.84	1.24
594	*s* [*psi*$^-$]	2.64	2.24
596	*u* [*psi*$^-$]	1.13	2.15
604	*sh* [*psi*$^-$]	4.06	3.09
606	*uh* [*psi*$^-$]	2.83	2.48
614	*us* [*psi*$^-$]	3.55	2.98
626	*suh* [*psi*$^-$]	2.84	3.54
706	WT [*PSI*$^+$]	33.0	30.0
748	*suh* [*PSI*$^+$]	34.2	34.1
600	*b* [*psi*$^-$]	0.95	1.12
598	*c* [*psi*$^-$]	1.18	1.08
617	*bc* [*psi*$^-$]	1.11	1.31
725	WT [*psi*$^-$] pCur1↑	0.34	
727	WT [*psi*$^-$] pBtn2↑	1.50	

The Dual-Glo Luciferase Assay System (Promega) was used with cells of the indicated genotype carrying pSC5 (*CEN LEU2* Renillia luciferase–UAA–Firefly luciferase) (65) and processed as described (44). * The ratio of Firefly/Renilla of the WT in experiment 1 was set to 1.00 for ease of comparison. b, *btn2*Δ; c, *cur1*Δ; h, *hsp104*^{T160M}; s, *ssz1*Δ; u, *upf1*Δ.

*btn2*Δ *cur1*Δ mutations decrease [*PSI*$^+$] generation, opposite to their effect on [URE3] previously described. Second, the increased frequencies of [*PSI*$^+$] appearance in strains defective for all three of the antiprion systems *ssz1*Δ, *upf1*Δ, and *hsp104*^{T160M} are much more than the sum of the single-mutant frequencies. We explore the second of these effects first, limiting our attention to *ssz1*Δ, *upf1*Δ, and *hsp104*^{T160M}.

Compared with [*PSI*$^+$], Antiprion Mutations Only Slightly Affect Nonsense Codon Read Through. Since *upf* and ribosome-associated chaperone gene (e.g., *ssz1*) mutations are known to have minor effects on read through of nonsense codons, it was possible that combinations of these mutations might have major effects that would obscure effects on generation or propagation of [*PSI*$^+$] itself. However, we found that [*PSI*$^+$] increased read through 33-fold in the wild type, but even the triple *ssz1*Δ, *upf1*Δ, and *hsp104*^{T160M} [*psi*$^-$] mutant only gave a 3-fold effect (Table 2). Moreover, the *ssz1*Δ *upf1*Δ *hsp104*^{T160M} [*PSI*$^+$] was indistinguishable in read through from the wild-type [*PSI*$^+$] (Table 2). The *btn2*Δ, *cur1*Δ, and *btn2*Δ *cur1*Δ [*psi*$^-$] mutations did not affect read through (Table 2). Overexpression of either Btn2 or Cur1 in the wild-type [*psi*$^-$] host had only small effects compared with the effect of [*PSI*$^+$] (Table 2). Barbitoff et al. (21) report that overexpression of Cur1 increases read through 1.5-fold, but overexpression of Btn2 had no effect.

Many [*PSI*$^+$] Variants Are Destabilized by Several Antiprion Systems. The elevation in [*PSI*$^+$] or [URE3] generation frequency in the single mutants is due to 1) some variants that cannot propagate in the wild type but can in the single mutant, meaning that the normal protein blocks propagation of those variants but the other antiprion systems are intact in the mutant and cannot block those variants, and 2) prion variants that can propagate in the wild type are generated more frequently in the mutant. If the antiprion components act independently in these processes and there are no novel variants emerging, then the frequencies in the multiple mutants should be additive or less than additive (see above). However, the observed frequencies in the *suh* strains are more than 5- (Table 1) or 10-fold (Table 3) higher than the sum of the frequencies of *s*, *u*, and *h*. The synergistic effect of mutations in multiple antiprion components is true for spontaneous prion generation and for prion generation induced by overproduction of the prion protein (Table3). We have observed frequencies as much as 5,000-fold above the wild-type rate (Table 3). We infer that there must be prion variants arising in the multiple mutants that can be cured or whose generation can be prevented by any of two or more of these antiprion components. To test this inference, we examined individual [*PSI*$^+$] variants originally isolated in an *suh* host for their ability to propagate when one or more antiprion components are restored. Among 70 [*PSI*$^+$] isolates generated in *suh* strain MS626, 8 were as stable when mated with a wild type as when mated with another *suh* strain. Another 20 of the 70 isolates were relatively stable in the wild type, while 3 were unstable even in an *suh* host. Among the remaining 39 isolates, 23 were examined by cytoduction (cytoplasmic mixing) (Table 4). All were essentially 100% stable when transferred by cytoduction to another *suh* strain but completely lost on transfer to a wild-type host (Table 4 and *SI Appendix*, Table S4). These 23 variants were each transferred by cytoduction to combinations of *s*, *u*, and *h* and the stability of the prion measured. Nearly all of these variants are sensitive to more than one antiprion system. For example, transfer to the *sh* host restores only *U*, and nearly all of the variants are strongly destabilized. Likewise, transfer to the *u* host, restoring both *S* and *H*, destabilizes each of these same variants to some extent (Table 4 and *SI Appendix*, Table S4). Examination

Table 3. De novo generation of [*PSI*$^+$]

Host	Spontaneous, [*PSI*$^+$]/10^6 cells	Induced, [*PSI*$^+$]/10^6 cells
WT	0.3 ± 0.6	11 ± 2
hsp104^{T106M}	3 ± 0.6	570 ± 120
*ssz1*Δ	13 ± 2	1,630 ± 190
*upf1*Δ	13 ± 5	1,830 ± 30
*ssz1*Δ *hsp104*^{T106M}	490 ± 33	44,800 ± 5,700
*upf1*Δ *hsp104*^{T106M}	470 ± 27	60,000 ± 21,000
*upf1*Δ *ssz1*Δ	480 ± 43	46,000 ± 3,200
*ssz1*Δ *upf1*Δ *hsp104*^{T106M}	520 ± 50	49,000 ± 2,300

Data are the mean of three determinations ± SD. "Induced [*PSI*$^+$]" means [*PSI*$^+$] generation produced by overexpression of Sup35NM, which includes the prion domain.

Table 4. Sensitivity of [*PSI*$^+$] isolated in the *suh*Δ strain to the replacement of combinations of antiprion systems

Donor	Recipient	Diploid, % Ura+ (isolates/total)	Cytoductant, % Ura+ (isolates/total)
*suh*Δ [*PSI*$^+$]	WT ρ^o	~0	~0
	h ρ^o	0–20	0–10
	s ρ^o	0–40 (14/23) >80 (8/23)	20–40 (13/23) 50–70 (6/23)
	u ρ^o	90–100	60–70 (13/22) 80–90 (6/22)
	sh ρ^o	20–40 (10/22) 90–100 (8/22)	10–30 (11/22) 40–60 (7/22)
	uh ρ^o	<30 (6/11)	>50 (4/15) 0–10 (6/15)
	us ρ^o	~0–10 (13/22) 40–50 (2/22) 80–100 (6/22)	N/A
	suh ρ^o	~100	~100

Among 70 [*PSI*$^+$] isolates arising in the *suh*Δ strain MS626, 39 were found to be stable when mated with another *suh*Δ strain but completely lost on mating with a wild type. Cytoduction into strains carrying all combinations of *upf1*Δ (*u*), *ssz1*Δ (*s*), and *hsp104*^{T160M} (*h*) were carried out, and the phenotypes of cytoductants and diploids formed were recorded (*SI Appendix*, Tables S4 and S5 has detailed results). The percentages of Ura+ in diploids and cytoductants are shown, and the fractions of isolated prion variants with the given percentages of Ura+ are in parentheses, except when all isolates had the given percentage of Ura+.

of the diploids formed in these experiments, in which the restored genes are heterozygous and so, possibly expressed below normal levels, shows similar results (*SI Appendix*, Table S5). The same conclusion is obtained by correcting single-antiprion defects with a centromere-containing (CEN) plasmid encoding the normal gene with its own promoter in each of 11 prion variants isolated in an *suh* strain (Table 5). In nearly every case, restoring any single-antiprion gene destabilizes each prion isolate (Table 5). These data (Table 5) confirm that it is indeed the *s*, *u*, and *h* defects that allow these variants to propagate and not some other putative adventitious mutations in some of the strains. One case, highlighted in *SI Appendix*, Table S5, is not well cured by restoring any single gene but is well cured by providing both *S* and *U*.

Multiple Previously Unknown [*PSI*+] Variants Arise in *suh* Strains. The frequency of prion loss of the isolates examined in Table 4 shows several cases in which the isolate stabilities fall into two clearly different patterns. For example, 8 isolates mated with the *ssz1*Δ strain show >80% [*PSI*$^+$] diploids (only 20% loss), while another 14 isolates show only 0 to 40% [*PSI*$^+$] diploids (60 to 100% loss) under these conditions. Mating with the *upf1*Δ strain results in only 0 to 20% loss for 6 isolates but 90 to 100% loss for 13 other isolates. These results are apparently due to prion variant differences among the isolates. Of course, none of these variants could be detected in single-antiprion mutants because all of these are sensitive to more than one system (Tables 4 and 5) and would be eliminated from the single mutants.

Increased Generation in *suh* Strains of [*PSI*$^+$] Variants Stable in Wild-Type Cells. Because 8 of 70 isolates in an *suh* host were stable in a wild-type host and the overall frequency of [*PSI*$^+$] is 230- to ~5,000-fold elevated in the *suh* host compared with the wild type (Tables 1 and 3), the generation of such typically studied [*PSI*$^+$] variants is increased between 25- and 500-fold in the *suh* strain [(8/70) × 230 or (8/70) × 5,000]. We infer that it is a combination of elevated generation of these [*PSI*$^+$] variants and the failure to cure [*PSI*$^+$] variants that are hypersensitive to just one antiprion system [as previously described (33, 38, 43)] plus the failure to cure the new variants that are sensitive to two or three antiprion systems that produces the extraordinarily high frequencies of [*PSI*$^+$] arising in the *suh* cells.

Btn2 and Cur1 Promote [*PSI*$^+$] Generation and Propagation. In otherwise wild-type cells, *b*, *c*, or *bc* mutations resulted in a lower frequency of [*PSI*$^+$] arising (Table 1). For *u* or *h* single mutants, their [*PSI*$^+$] generation frequency is elevated but is similarly reduced by added *b*, *c*, or *bc* mutations (Table 1). Another experiment focused on the wild type, in which the effects of *b*, *c*, or *bc* mutations on spontaneous [*PSI*$^+$] appearance or that induced by transient overproduction of Sup35NM from a *GAL1* promoter were observed (Table 6). The normal levels of both Btn2p and Cur1p are important for the appearance of [*PSI*$^+$] either spontaneously or following Sup35NM overproduction (Table 6).

[PIN$^+$] (for [*PSI*$^+$] inducibility) is a prion of Rnq1p that can rarely cross-seed the amyloid of Sup35p and is necessary for detectable [*PSI*$^+$] generation, whether spontaneous or induced by prion protein overproduction (45–47). We find that [PIN$^+$] obtained from the wild type and from the mutants with the highest [*PSI*$^+$] generation frequency have essentially the same [*PSI*$^+$] inducibility activity when assayed in the same wild-type strain (*SI Appendix*, Fig. S1). In addition, replacing *BTN2* and *CUR1* fully restores [*PSI*$^+$] generation frequency to a *btn2*Δ *cur1*Δ strain, indicating that [PIN$^+$] loss or "mutation" was not the cause of their [*PSI*$^+$] generation inability (*SI Appendix*, Table S3).

Hsp42 is necessary for curing of [URE3] by Btn2p or Cur1p, and overproduced Hsp42 also cures [URE3] in a process requiring Cur1 (but not Btn2) (19). However, we find that overproduced Hsp42 does not affect [*PSI*$^+$] generation in wild type (WT), nor does it restore [*PSI*$^+$] generation to *btn2*Δ or *cur1*Δ strains.

To distinguish effects on prion generation from prion propagation, nine [*PSI*$^+$] isolates obtained in a wild-type strain were transferred by cytoduction to wild-type, *cur1*Δ, *btn2*Δ, or *cur1*Δ *btn2*Δ recipients. All nine isolates were stably propagated in the wild-type recipient strain and in the heterozygous diploids

Table 5. Normal levels of Ssz1, Upf1 or Hsp104 can each cure previously unknown [*PSI*+] variants

Strain	No. of Ura+/total transformants			
	pRS313	p*SSZ1*	p*UPF1*	p*HSP104*
suh [*PSI*$^+$] 1	64/65	30/92****†	20/35**†	33/52**†
2	82/85	34/80****†	53/86**†	43/55*†
3	51/55	15/31*†	19/33*†	10/15†
4	18/35	11/27	3/6	29/38
5	84/90	54/100**†	cont.	46/59
6	50/52	25/51**†	39/84**†	68/76†
7	74/75	56/102***†	24/59****†	65/84*†
8	78/80	cont.	8/34****†	cont.
9	41/45	45/65†	57/97*†	53/58
10	37/38	19/33*†	10/15†	29/42*†
11	52/54	14/33**†	3/6†	13/15

Eleven [*PSI*$^+$] isolates generated in the *ssz1*Δ *upf1*Δ *hsp104*^{T160M} strain MS626 were chosen from among those that give all Ura+ diploids with the *ssz1*Δ *upf1*Δ *hsp104*^{T160M} strain MS627 and all Ura− diploids with a wild type. Each was transformed with vector (pRS313) or p*SSZ1*, p*UPF1*, or p*HSP104* CEN plasmids carrying the indicated gene with its own promoter. The fraction of transformants remaining [*PSI*$^+$] (as judged by the suppressible *ura3-14* allele on p1520) is shown. Ura− subclones of each case showed 80 to 100% Ura− after loss of each plasmid. cont., contaminated. *$P < 0.05$; **$P < 10^{-2}$; ***$P < 10^{-3}$; ****$P < 10^{-4}$.

†Destabilized by restoring the indicated gene (compared with the vector alone).

Table 6. De novo generation of [*PSI*$^+$] is promoted by Btn2p and Cur1p

Host	Spont. Ura+/10^6 cells	Guanidine curable	Spontaneous [*PSI*$^+$]/10^6 cells	Induced Ura+/10^6 cells	Guanidine curable	Induced [*PSI*$^+$]/10^6 cells
WT	2.5 ± 0.5	14/16	2.2	42 ± 7.5	21/24	37
*cur1*Δ	2.5 ± 0.5	2/16	0.3	24 ± 5.0	1/20	1
*btn2*Δ	3.0 ± 1.0	0/16	0	25 ± 2.5	0/16	0
*btn2*Δ*cur1*Δ	3.5 ± 1.5	0/16	0	24 ± 6.0	2/16	3

Serial dilutions of the indicated strains (WT = 5,956; *cur1*Δ = 5,965; *btn2*Δ = 5,977; *btn2*Δ*cur1*Δ = 5,974) were plated on −Ura or −Ade plates, and colonies were counted after 7 d. A sample of each group was checked for sensitivity to curing by 3 mM guanidine HCl on 1/2 YPD plates to distinguish prions (curable) from tRNA or other chromosomal suppressor mutations (not curable).

formed (as a side product) in the cytoduction experiments (Table 7). However, all nine isolates were destabilized in the *cur1*Δ, *btn2*Δ, or *cur1*Δ *btn2*Δ cytoductants (Table 7). To confirm that the Ura− phenotypes of these recipients were due to loss of the prion and not just loss of the phenotype, sample Ura− cytoductants from Table 7 were used as donors to return the cytoplasm to a [*psi*$^-$] version of the wild type (*SI Appendix*, Table S6). Nearly all of these "return cytoductants" were Ura−, indicating that the prion was lost in the Ura− *cur1*Δ, *btn2*Δ, or *cur1*Δ *btn2*Δ cytoductants (*SI Appendix*, Table S6). To confirm that the loss of [*PSI*$^+$] was due to the loss of Cur1 and/or Btn2, [*PSI*$^+$] variants were isolated in *cur1*Δ, *btn2*Δ, or *cur1*Δ *btn2*Δ strains, each complemented with a CEN plasmid carrying the wild-type genes under their native promoters (*SI Appendix*, Table S7). In most cases, the [*PSI*$^+$] variants were stable as long as the complementing plasmid remained but were destabilized once the complementing plasmid was lost (*SI Appendix*, Table S5). As expected from previous results (20, 24), each of the three [*PSI*$^+$] variants isolated in a wild-type host were stable when transferred to WT, Btn2p overproducing, Cur1p overproducing, or cells overproducing both (*SI Appendix*, Table S8).

Deficiency of Btn2p and/or Cur1p impairs [*PSI*$^+$] generation in wild-type cells and in some of the *ssz1*Δ, *upf1*Δ, and *hsp104*^{T160M} single and double mutants but not in the triple-mutant *ssz1*Δ *upf1*Δ *hsp104*^{T160M} host or in *ssz1*Δ *hsp104*^{T160M} cells (Table 1 and *SI Appendix*, Table S3). This effect and the disparity between the effects of Btn2 and Cur1 on [*PSI*$^+$] and [URE3] are discussed below.

Seed Number Effect of Btn2p and Cur1p on [*PSI*$^+$]. The number of infectious prion particles can be measured by arresting filament scission by Hsp104 by growing colonies on 3 mM guanidine and counting the number of Ade+ (mostly [*PSI*$^+$]) cells in a clone (48). Normal levels of Btn2p and Cur1p selectively cure [URE3] prion variants with low seed numbers (19). Although both proteins promote rather than cure [*PSI*$^+$], we examined their effect on the [*PSI*$^+$] seed number (Fig. 1). Transfer of a [*PSI*$^+$] with seed number ∼160 from a wild-type strain to *cur1*Δ, *btn2*Δ, or *cur1*Δ *btn2*Δ strains showed a sharp drop in seed number, consistent with the instability of [*PSI*$^+$] in such strains. In contrast, transfer to cells overproducing Btn2p, Cur1p, or both resulted in an increase in seed number. Similar results were also obtained with standard strong or weak variants of [*PSI*$^+$] (Fig. 1).

When [*PSI*$^+$] was returned from wild-type, *cur1*Δ, or *btn2*Δ *cur1*Δ [*PSI*$^+$] cytoductants to a [*psi*$^-$] version of the original wild type, the seed number returned to its original value. We could not recover any stable [*PSI*$^+$] *btn2*Δ cytoductants to use in this experiment. On returning [*PSI*+] to the wild type from the hosts overproducing Btn2p, Cur1p, or both, the seed number remained elevated, suggesting that this environment had selected or produced a changed variant of [*PSI*$^+$]. This finding is reminiscent of those of Lancaster et al. (49) that in several Hsp90-related mutants, [PIN$^+$] changes its character in a way that is maintained on returning to a wild-type environment.

Table 7. [*PSI*$^+$] isolates in WT cells were unstable in *cur1*Δ, *btn2*Δ, and *btn2*Δ*cur1*Δ mutants

Donor	Recipient	Ura+ diploids per total	Ura+ cytoductants per total
WT [*PSI*$^+$] 1–9	WT ρ^o	10/10	8/10
		10/10	10/10
		10/10	9/10
		—	—
		10/10	9/10
		10/10	10/10
		10/10	10/10
		10/10	10/10
		10/10	8/10
	*cur1*Δ ρ^o	8/10	6/9
		10/10	4/10
		9/10	6/10
		8/10	4/13
		9/10	3/12
		3/9	3/8
		9/10	2/10
		9/10	2/14
		10/10	0/3
	*btn2*Δ ρ^o	10/10	4/10
		9/10	3/10
		10/10	2/10
		10/10	4/15
		9/10	2/15
		10/10	4/15
		9/10	2/11
		10/10	5/16
		10/10	2/10
	*btn2*Δ*cur1*Δ ρ^o	7/10	0/10
		10/10	2/10
		10/10	3/10
		10/10	1/14
		9/10	3/15
		10/10	3/15
		10/10	4/15
		10/10	2/15
		10/10	2/15

WT [*PSI*$^+$] isolate (stable Ura+ and curable by 5 mM GdnHCl) → WT, *cur1*Δ, *btn2*Δ, *btn2*Δ*cur1*Δ. Stable guanidine-curable [*PSI*$^+$] isolates in WT MS659 were transferred by cytoduction (cytoplasmic mixing) from isolates MS737 to MS745 into strains MS710 (WT), MS712 (*btn2*Δ), MS711 (*cur1*Δ), and (*btn2*Δ*cur1*Δ). Ura+ and Ura− cytoductants of each were used as "reverse-cytoduction" donors into WT strain MS714 (*SI Appendix*, Table S6).

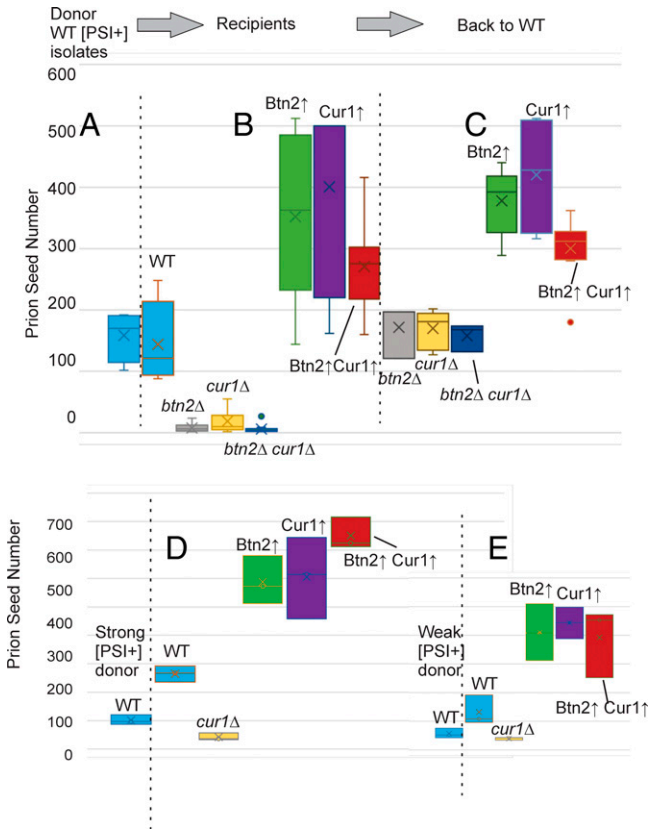

Fig. 1. [*PSI*$^+$] seed number is elevated by passage through cells overproducing Btn2p or Cur1p. (*A*) Three [*PSI*$^+$] isolates in WT MS681 were transferred by cytoduction to strains MS746 (WT), MS711 (*cur1*Δ), MS712 (*btn2*Δ), MS713 (*btn2*Δ *cur1*Δ), MS725 (WT pM94 [*2* μ *URA3* P_{CUR1}*:CUR1*]), MS727 (WT pM93 [*2* μ *URA3* P_{ADH1}*:BTN2*]), and MS729 (WT pM95 [2 μ *LEU2* P_{BTN2}*:BTN2* P_{CUR1}*:CUR1*]). Seed numbers were measured for 4 to 13 [*PSI*$^+$] cytoductant clones of each (*B*). Then, [*PSI*$^+$] cytoductants (except for *btn2*Δ, for which there were no [*PSI*$^+$] cytoductants) were used as donors to transfer [*PSI*$^+$] back to a [*psi*$^-$] version of the original [*PSI*$^+$] donor. The seed numbers of these "back cytoductants" were then determined (*C*). Seed number values of cytoductants from standard strong (MS224; *D*) and weak (MS225; *E*) [*PSI*$^+$] strains to the same w.t.; *cur1*Δ; and strains overproducing Cur1, Btn2, or both as used in *A* and *B*. No cytoductants in the *btn2*Δ recipient were obtained.

Discussion

Here, we report an exceptionally high frequency (up to 5,000-fold above wild-type levels) (Table 3) of the spontaneous emergence of the [*PSI*$^+$] prion in *upf1Δ ssz1Δ hsp104*^{T160M} strains, defective in three antiprion systems active against the [*PSI*$^+$] prion. Single mutants in any one of these genes develop prions at about 10 to 15 times the normal rate, largely by producing [*PSI*$^+$] variants sensitive to curing by normal levels of the missing protein. Those prion variants are resistant to the other two systems, which are intact in the single mutant. However, many of the variants arising in the triple mutant are destabilized by replacement of any one of the three genes. The curing of these [*PSI*$^+$] variants by replacing only a single antiprion gene also proves that these three systems can work independently of each other. Moreover, these previously unknown [*PSI*$^+$] variants are cured by normal levels of the Hsp104 disaggregase, by the ribosome-associated chaperones (Zuo1p, Ssb1/2, and Ssz1p), or by the NMD proteins (Upf1,2,3) that normally form a complex with Sup35p. In this sense, the different systems cooperate to limit prion formation.

In addition to allowing propagation of these easily cured [*PSI*$^+$] variants, the frequency of generation of the [*PSI*$^+$] variants that are stable in a normal strain is elevated 25- to 500-fold. It has been previously shown that the Upf1 proteins, the ribosome-associated chaperones (including Ssz1p), and Hsp104 keep down the frequency of the generation of these prions by less than the 10- to 15-fold overall increase of [*PSI*$^+$] generation of these mutants (30, 33, 38, 43). This limitation of [*PSI*$^+$] generation may also consist of the elimination of some early intermediate on the way to prion formation, in effect also constituting a "curing" of sorts.

The extremely high frequency of [*PSI*$^+$] generation in the *upf1Δ ssz1Δ hsp104*^{T160M} triple mutants indicates that prion formation is not really a rare event. Rather than arising in about 1 or a few per 10^6 cells, [*PSI*$^+$] is formed in at least 1 or a few in 1,000 cells, but it is usually cured (Fig. 2). This picture resembles the role the multiple DNA repair systems play in reversing, excising, or recombining to replace the many forms of DNA damage that occur at high frequency many thousands of times the frequency of mutations that become fixed. Because prions are heritable, they are protein genes playing a role like the mutant form of a DNA gene.

The antiprion systems have considerable specificity, with Btn2p and Cur1p curing [URE3] (20) and an unrelated artificial prion (23) but having the opposite effect on nearly all [*PSI*$^+$] variants. The ribosome-associated chaperones so important for [*PSI*$^+$] do not detectably affect [URE3] (33), although their general function in assuring proper folding of nascent proteins led us to expect that they would affect all prions involving misfolding. The normal complex of Upf proteins with Sup35p seems to be the basis of its antiprion action, and so, they should be [*PSI*$^+$] specific and do show an Sup35p-specific inhibition of amyloid formation (43). The Hsp104 prion-curing activity is most active on [*PSI*$^+$] but does inefficiently cure [URE3] as well (20, 50). While there are prion variants specifically hypersensitive to just one of Ssz1, Upf1, or Hsp104, there are others, which we describe here, that are sensitive to all three, indicating that there is some breadth to the scope of each system.

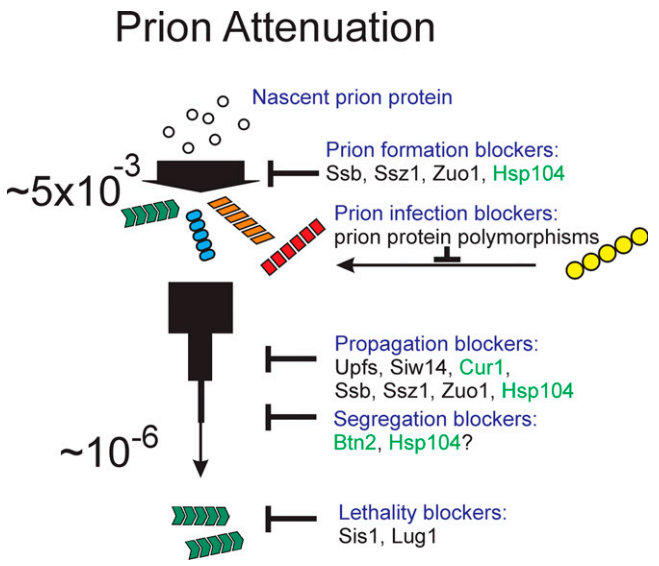

Fig. 2. Prion attenuation at multiple levels. Prion generation, infection, propagation, segregation, and pathology are each blocked by one or more systems. We show that in a strain defective in several antiprion systems, [*PSI*$^+$] can arise at as much as 5,000-fold the rate in a wild-type strain. Proteins shown in green have both antiprion and proprion functions. Hsp104 breaks filaments, forming new growing ends, while we show here that Cur1 and Btn2 promote [*PSI*$^+$], possibly at both generation and propagation, although they impair [URE3] propagation (see Fig. 3).

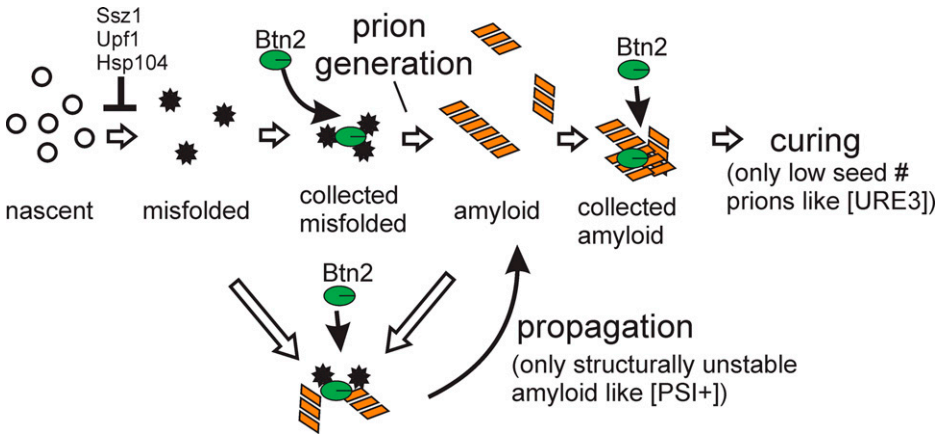

Fig. 3. Model to explain prion generation and propagation data in terms of protein interactions. Btn2p is known to collect prion amyloid filaments (20) and other misfolded proteins (22, 55). We suggest that Btn2 promotes [*PSI*$^+$] generation by collecting misfolded Sup35p molecules. [*PSI*$^+$] generation is lowered by *btn2*Δ or *cur1*Δ mutations in w.t. or most single and multiple mutants of anti-[*PSI*$^+$] genes but not in *upf1Δ ssz1Δ hsp104*^{T160M} or *ssz1Δ hsp104*^{T160M} strains, perhaps because the latter have very high levels of misfolded Sup35p. Prion curing by normal levels of Btn2 acts only on variants with lower seed number, and [*PSI*$^+$] prions generally have higher seed number than those of [URE3] and so, are insensitive to Btn2 curing. Btn2 may promote [*PSI*$^+$] propagation by bringing together misfolded Sup35 molecules and [*PSI*$^+$] amyloid filaments. Sup35 filaments are structurally much less stable than Ure2 filaments and so, may need Btn2's help to increase the local concentration of monomer. Ssz1p, Upf1p, and Hsp104p doubtless act at other steps in addition to preventing or reversing misfolding of nascent proteins.

How can we explain the difference between the curing action of Btn2 and Cur1 on [URE3] and the pro-[*PSI*$^+$] action of both proteins? Barbitoff et al. (21) have found that overproduced Cur1p (but not Btn2p) increases read through of termination codons in [*PSI*$^+$] cells but not if they are [*psi*$^-$]. Usually, this strengthening of the [*PSI*$^+$] phenotype is accompanied by smaller filament size, but Barbitoff et al. (21) found no size change by partial denaturing gels for either strong or weak variants. These authors support the view of Malinovska et al. (23) that overexpressed Cur1p or Btn2p binds to the Hsp40 family member Sis1p, moves it into the nucleus, and thereby, depletes cytoplasmic Sis1p, resulting in [URE3] prion loss but not [*PSI*$^+$] because the latter's requirement for Sis1p is not as stringent. As we have discussed elsewhere (19), we doubt that the effects of Cur1p or Btn2p on Sis1p can explain the results. In normal cells, Sis1p is 450- and 300-fold more abundant than Cur1p and Btn2p, respectively, so they are unlikely to bind much of the Sis1p. Even when Cur1p or Btn2p is overproduced, it increases the nuclear concentration of Sis1p by less than two-fold (23); because the nucleus is only about 1/8 the volume of the cytoplasm, it can only be decreasing the cytoplasmic levels of Sis1p by less than 1/8, a change that would not cure [URE3] (51). The Sis1p model also does not explain the colocalization of Btn2p and Ure2p amyloid in cells being cured. Moreover, deleting the Btn2 nuclear localization sequence does not prevent curing of [URE3] (20) but does prevent the modest concentration of Sis1p in the nucleus (23). The role of Sis1p in [*PSI*$^+$] propagation is in helping the cleavage of filaments in cooperation with Hsp104 and Hsp70 (51–53). However, Barbitoff et al. (21) find there is no detectable alteration in the size of these filaments. The lowering of seed number expected if Sis1p was sequestered in the nucleus should make the [*PSI*$^+$] phenotype weaker, but it is reported to be stronger (21).

We find dramatic effects of *btn2*Δ and *cur1*Δ on both generation and propagation. Spontaneous generation was at least 10-fold below the wild type in deletion mutants, except those with both *ssz1*Δ and *hsp104*^{T160M} mutations. Propagation of most [*PSI*$^+$] variants isolated in a wild type are unstable in *btn2*Δ or *cur1*Δ mutants. These actions of Btn2 and Cur1 could be explained if these two proteins inhibit the antiprion activities of Ssz1 and of Hsp104, but Btn2 and Cur1 are found at only about 170 and 110 molecules per cell, respectively (27)(https://pax-db.org/species/4932), while there are 11,000 to 29,000 molecules of Hsp104 and 25,000 to 150,000 molecules of Ssz1 per cell. Alternatively, Btn2 and Cur1 may inhibit another anti-[*PSI*$^+$] system distinct from those examined here, but this explanation also requires new (unlikely) activities of Btn2 or Cur1.

The opposite effects of Btn2 and Cur1 on [URE3] and [*PSI*$^+$] may be better explained (at least for Btn2) based on the uniformly higher seed numbers of [*PSI*$^+$] prion variants compared with [URE3] variants (e.g., refs. 20 and 48) and the lower stability of Sup35p filaments (14) compared with those of Ure2p (54) (Fig. 2). Btn2 collects filaments and cures [URE3], with high–seed number variants only cured with overproduced Btn2, while low–seed number variants are cured by the normal level of Btn2 (19). Although Btn2 partially colocalizes with Sup35p amyloid in a [*PSI*$^+$] strain, it does not cure most [*PSI*$^+$], presumably because [*PSI*$^+$]'s seed number is too high (20). Btn2 collects misfolded proteins (55), so Btn2 may collect misfolded Sup35p molecules in one place so they can more easily form amyloid filaments. Btn2 may do the same with misfolded Ure2p, but it cures most prions formed because their seed number is generally lower. By collecting misfolded Sup35p and Sup35p amyloid at the same site, Btn2 may facilitate filament elongation and thus, prion propagation as well. The Ure2p prion domain forms much more stable structures than does Sup35 and may not need this help. This model (Fig. 3) may explain the action of Btn2 and requires no new assumptions to explain the results.

Plainly, cells do not want to have [*PSI*$^+$] prions around; they block >99% of those that could arise. The antiprion systems are precisely analogous to DNA repair since prions are heritable and so, act as genes. Our findings are of wide interest because in addition to the rare human the classic mammalian prion protein (PrP)-related prion diseases, the very common amyloidoses Alzheimer's disease, Parkinson's disease, type II diabetes, and others have prominent prion aspects, including frank infectivity (56–60), and are largely untreatable at this time. It is hoped that human analogs or homologs of the yeast antiprion systems can be found and manipulated to treat these diseases.

Methods

Nomenclature. Yeast prions (e.g., [*PSI*$^+$] or [PIN$^+$]) are shown in brackets, indicating that they are nonchromosomal genetic elements.

Strains and Media. Strains used in this study are listed in *SI Appendix*, Table S1. The series of mutants defective in various antiprion systems was generated by multiple crosses with strains 4839 (BY4742 background) and 5385 (779-6A background) and further confirmed using PCR amplification (61). Media used were as described by Sherman (62). Induction of *GAL1*-promoted SUP35 (prion domain NM) was conducted using galactose–raffinose-containing media as previously described (43). For scoring [*PSI*$^+$], the chromosomal suppressible *ade1-14* allele or *ura3-14* (12) on p1520/pM6 [*CEN LEU2 ura3-14 GAL1*$_{promoter}$*-SUP35NM* (44)] was used. For [*PSI*$^+$] scoring experiments using p1520/pM6, the plasmid was maintained in strains/colonies without leucine to prevent loss of this plasmid. The 1/2 YPD medium contains (per liter) 5 g yeast extract, 20 g peptone, 20 g dextrose and 20 g agar.

Plasmids. Plasmids used in this study are listed in *SI Appendix*, Table S2. All plasmids were generated previously, and their effects were confirmed in previous studies (19, 33, 43, 44). An *ADH1*-promoted Rnq1p-GFP–expressing plasmid was generated previously (63).

[*PSI*$^+$] Generation. Yeast strains were inoculated into 3 mL of synthetic complete (SC) glucose/galactose and raffinose liquid media and cultured for 2 d at 30 °C. Cells were diluted to 10^7 cells/mL and spread in 10-fold dilutions on standard SC plates lacking uracil (using *ura3-14*) or adenine (using *ade1-14*). Ura+/Ade+ colonies were counted after 5 to 7 d of incubation at 30 °C. A calculated 10^2 cells from each strain were spread on yeast extract – peptone – adenine - dextrose medium (YPAD) media to check the accuracy of dilutions and the viability of cells. A number of Ura+/Ade+ isolates were confirmed as [*PSI*$^+$] based on their curability on 3 or 1 mM guanidine (64).

Dual Luciferase Assay. pSC5 (Table S2) was used to measure translation termination efficiency of strains. This plasmid has the upstream *Renilla Luc* fused with a UAA stop codon in frame to the downstream firefly *Luc* (65). The activity of each luciferase was measured separately in the same sample using the Dual-Glo Luciferase Assay System (Promega) and a Berthold Luminometer (Titertek Berthhold). Samples were prepared as described previously (44).

Cytoduction. Cytoduction experiments, the transfer of cytoplasmic elements from donor strains to recipient strains, were performed as described previously (66). Briefly, a ρ+ donor is mixed in modest excess with a ρ^0 recipient, and mating proceeds on YPAD at 30 °C. When donor and recipient strains are both *ssz1*Δ, increased mating time (from 7 to 24 h) on YPAD produces enough cytoductants for analysis. Mating mixtures are then plated on media selecting against the donor, and colonies are tested on dropout plates and glycerol plates to distinguish diploids from cytoductants (recipients that have received cytoplasm from the donor and so, can grow on glycerol) and from unmated cells.

Measuring [*PSI*$^+$] Prion Seed Number. Following Cox et al. (48) and our previous studies (43), freshly grown [*PSI*$^+$] strains were streaked for single colonies on YPAD containing 5 mM guanidine HCl. After 2 or 3 d, single colonies were isolated with the underlying agar block, suspended in sterile water, and plated on prion-selecting media (−Ade plate). The number of Ade+ colonies arising was counted and assumed to indicate the seed number of the [*PSI*$^+$] prion in the founder cell. For each strain, at least six individual colonies were tested. A number of Ura+/Ade+ isolates were confirmed as [*PSI*$^+$] based on their curability on 3 or 1 mM guanidine.

Statistical Analysis. In most cases, the SD was determined from multiple results using the STDEV() function of Excel. In other cases, data were assumed to follow the binomial distribution because each data point expresses two alternative results: a cell becoming [*PSI*$^+$] or not. The number of total cells tested (N) and the probability of a [*PSI*$^+$] (p) or [*psi*$^-$] (q) are used in calculations. The results should be approximately normally distributed as long as $Npq \gg 1$, which was true for all of our data. To calculate the probability that two sets of data could be samples from the same distribution of insertions, differing only because of random fluctuations on sampling, the two populations were combined, and $p_{tot} = (N_1p_1 + N_2p_2)/(N_1 + N_2)$. In the null case, we expect N_2p_2 to be close to N_2p_{tot}. The probability of a given difference $(N_2p_2 - N_2p_{tot})$ divided by the SD for the binomial distribution $((N_1 + N_2)p_{tot}(1 - p_{tot}))^{1/2}$ is set equal to z of the normal distribution and the probability obtained from a table.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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