[PSI+] prion propagation is controlled by inositol polyphosphates

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The yeast prions [PSI+] and [URE3] are folded in-register parallel β-sheet amyloids of Sup35p and Ure2p, respectively. In a screen for antiprion systems curing [PSI+] without protein overproduction, we detected Siw14p as an antiprion element. An array of genetic tests confirmed that many variants of [PSI+] arising in the absence of Siw14p are cured by restoring normal levels of the protein. Siw14p is a pyrophosphatase specifically cleaving the β phosphate from 5-diphosphoinositol pentakisphosphate (5PP-IP_5), suggesting that increased levels of this or some other inositol polyphosphate favors [PSI+] propagation. In support of this notion, we found that nearly all variants of [PSI+] isolated in a WT strain were lost upon loss of *ARG82*, which encodes inositol polyphosphate multikinase. Inactivation of the Arg82p kinase by D131A and K133A mutations (preserving Arg82p's nonkinase transcription regulation functions) resulted the loss of its ability to support [PSI+] propagation. The loss of [PSI+] in *arg82*Δ is independent of Hsp104's antiprion activity. [PSI+] variants requiring Arg82p could propagate in *ipk1*Δ (IP_5 kinase), *kcs1*Δ (IP_6 5-kinase), *vip1*Δ (IP_6 1-kinase), *ddp1*Δ (inositol pyrophosphatase), or *kcs1*Δ *vip1*Δ mutants but not in *ipk1*Δ *kcs1*Δ or *ddp1*Δ *kcs1*Δ double mutants. Thus, nearly all [PSI+] prion variants require inositol poly-/pyrophosphates for their propagation, and at least IP_6 or 5PP-IP_4 can support [PSI+] propagation.

prion | inositol polyphosphate | Arg82 | Siw14 | [PSI+]

There are a multitude of antiviral and antibacterial systems to deal with the variety of these infectious agents. *Saccharomyces cerevisiae* has at least nine proteins capable of forming prions, most based on amyloid filaments formed from normally nonamyloid proteins (reviewed in ref. 1). [PSI+] is a prion of the translation termination factor Sup35p, and [URE3] is a prion of the nitrogen catabolite repression factor Ure2p (2). These two prions are detected by phenotypes due to the partial deficiency of the active normal form of the protein. [PSI+] and [URE3] are based on amyloid filaments of Sup35p and Ure2p, respectively (3–11). Their folded in-register parallel β-sheet architecture (12–14) naturally suggests a mechanism by which the molecules in the filament transmit their conformation to monomers newly joining the chain by a type of templating (1, 15), in analogy to DNA transmitting its sequence to a newly forming chain. Distinct self-propagating amyloid conformations are believed to determine the many different prion variants that one can observe for a given prion protein sequence (9, 10, 16).

The newly formed [PSI+] and [URE3] prions are most often toxic or even lethal (17), and the infrequent occurrence of even their mildest forms (18–20) in wild strains indicates that they are, on the net, detrimental (19, 21; reviewed in ref. 22). One expects that there should be antiprion systems that prevent prion formation or cure them as they arise. Ssb1p and Ssb2p are ribosome-associated Hsp70 chaperones believed to assist the cotranslational folding of nascent proteins (23). In the absence of Ssb1/2, the frequency of [PSI+] generation is elevated (24). Restoring Ssb1 to the double mutant that has become [PSI+] does not cure the prion, indicating that the Ssb chaperones partially prevent [PSI+] from arising (24). The Hsp104 disaggregating chaperone is necessary for the propagation of most amyloid-based yeast prions but if overproduced can cure the [PSI+] prion (25–27). Mutation of the Hsp104 N-terminal domain eliminates its ability to cure [PSI+] by overproduction without affecting its prion propagation activity (28). Using this finding, we showed that this prion-curing activity of Hsp104 acts at normal levels of the protein to eliminate most spontaneous [PSI+] variants as they arise (29). Overproduction of Btn2p and Cur1p each cure the [URE3] prion (30), but normal levels of either protein cure most variants of [URE3] arising in their absence (31). Btn2p acts by collecting prion amyloid filaments at one place in the cell, so that one of the daughter cells is likely to be prion-free (30). These represent three (or four) antiprion systems working in normal cells to prevent prion generation or to cure newly arising prions. Mutation of each of these systems elevates spontaneous prion generation frequency by 10-fold or more.

Based on this experience, we devised a general screen for antiprion systems and found that Siw14p acts as an antiprion element. Siw14 is a pyrophosphatase specific for 5PP-IP_5 (5-diphosphoinositol pentakisphosphate) (32). Inositol polyphosphates (IPs) and pyrophosphates are signaling molecules regulating energy balance, phosphate uptake, DNA damage repair, telomere shortening, response to certain stress conditions, vesicle trafficking, and other functions (33). We further show that IPs are important for the propagation of most [PSI+] variants and that the Siw14 pyrophosphatase acts as a [PSI+]-curing factor by limiting the levels of some inositol poly-/pyrophosphates. The pathways of IP synthesis are shown in Fig. 1*A* (reviewed in refs. 3, 4, and 35).

Results

Isolation of Anti-[PSI+]–Defective Mutants. In designing a general screen to find [PSI+]-curing systems that are effective without protein overproduction, we assumed that such a system would be variant-specific because [PSI+] variants do arise despite such

Significance

The [PSI+] prion of baker's yeast is a filamentous polymer (amyloid) of the Sup35 protein, producing readthrough of translation termination and different degrees of growth slowing, depending on the prion variant. We show that certain inositol polyphosphates and pyrophosphates promote the propagation of the [PSI+] prion and that an inositol pyrophosphate pyrophosphatase has an antiprion effect. Inositol poly-/pyrophosphates are intracellular signaling molecules not previously connected with any amyloidosis.

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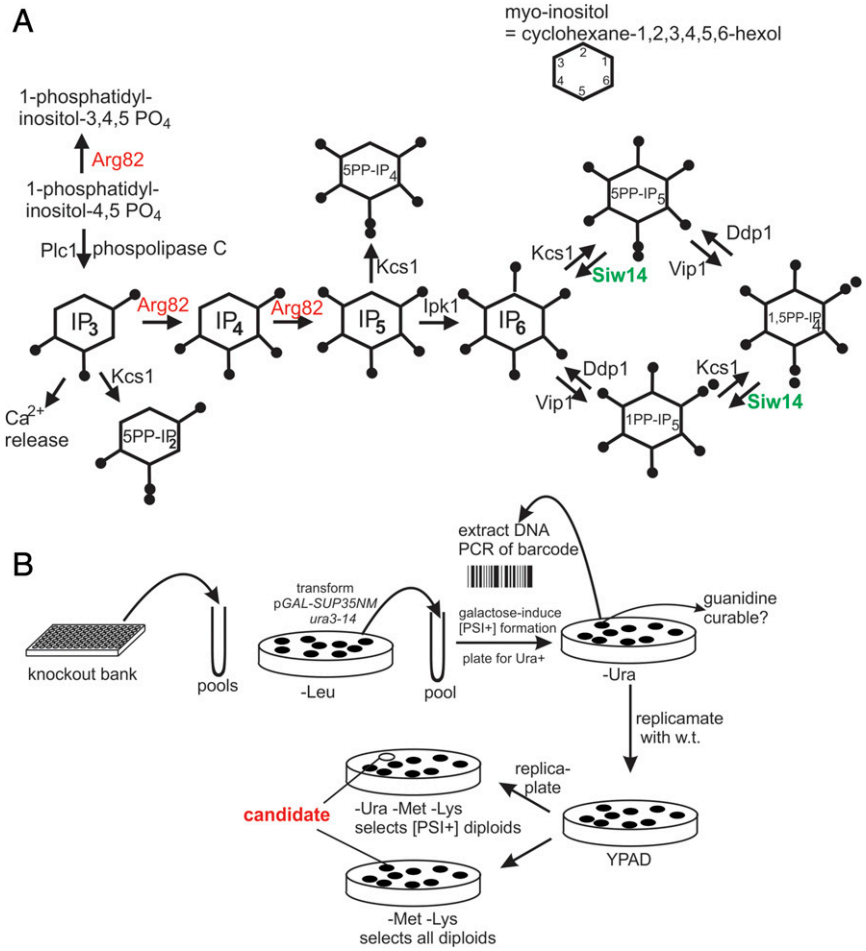

Fig. 1. (*A*) IP synthesis pathways. (*B*) Diagram of the isolation scheme for mutants in antiprion genes.

systems. Modeling our screen on the Btn2/Cur1 experiments, we generated [PSI+] variants in pools of subsets of the yeast knockout collection (36). To score [PSI+] in the knockout bank strains (all *ura3*Δ), we used *ura3-14* (37), a [PSI+]-suppressible nonsense allele, on a *CEN* plasmid (p1520) that includes the part of *SUP35* encoding the prion domain (NM) under control of the *GAL1* promoter (see Fig. S1 and Dataset S1). Overproduction of the amyloid-forming part of a prion protein (Sup35NM in this case) dramatically increases the frequency of prion formation (2). [PSI+] cells are Ura+, and [psi−] cells are Ura−. Pools of the *MAT***a** knockout bank were transformed en masse with p1520, grown for 24 h in galactose to induce the appearance of an array of [PSI+] variants, and plated on medium lacking Ura (−Ura) to select [PSI+] clones (Fig. *1B*). These clones were replica-mated with an isogenic WT *MATα* strain to complement the knockout of each clone. We looked for clones that formed diploids but not [PSI+] (i.e., Ura+) diploids. [PSI+] is efficiently cured by growth in the presence of 5 mM guanidine (38), a specific inhibitor of the disaggregating chaperone Hsp104 (39–41). We confirmed that the candidate haploid clones were [PSI+] by showing they were curable by guanidine and, as shown below, are transferred by cytoplasmic mixing (cytoduction). To identify the gene deleted in candidate anti-[PSI+]–defective clones, we amplified by PCR and sequenced the bar-code region of the *kanMX* module (42). Two such isolates, PB7 and PB14, carried *siw14::kanMX* and are characterized here.

PB7 was cured of [PSI+] and [PIN+] (a prion of Rnq1p needed for [PSI+] induction; see refs. 43–45) by growth on 5 mM guanidine, and then [PIN+] was replaced by cytoduction from strain 4457. [PSI+] generation was again induced by overproduction of Sup35NM in galactose medium, and Ura+ clones were isolated and again tested by guanidine curing and mating with the isogenic WT strain 4729. Nine of seventeen Ura+ guanidine-curable clones produced Ura− diploids on mating with 4729, suggesting that about half of the [PSI+] variants arising in the *siw14*Δ strain were cured by restoring normal amounts of Siw14p. We call such a Siw14p-sensitive prion variant "[PSI+ss]."

[PSI+ss] Is Stable in *siw14::kanMX* Strains and Is Lost When *SIW14* Is Restored. Eight new apparently [PSI+ss] isolates were obtained as above in the *siw14*Δ strain PB7 and were either subcloned on YPAD medium or were mated with isogenic WT strain 4729 and the diploids formed were subcloned on YPAD medium. Both the *siw14*Δ haploid and *siw14*Δ/+ diploid subclones were replica-plated to –Ura to test the stability of [PSI+] (Table 1). Each [PSI+ss] variant was more stable in the *siw14*Δ haploid than in the complemented (heterozygous) diploid.

The Ura− Phenotype in *SIW14* Hosts Is due to [PSI+] Loss. If the Ura− phenotype of diploids formed by mating *siw14*Δ [PSI+ss] with a WT strain is due to the loss of [PSI+ss], then all meiotic segregants of such diploids should be Ura−, whether they are *siw14*Δ or *SIW14*. Indeed, Ura− diploids of PB7 × 4729 and PB14 × 4729 produced only Ura− meiotic segregants (12 tetrads each). However, if PB7 (*siw14*::*kanMX* [PSI+ss]) was mated with the isogenic WT [psi−] strain 4729 (= 4813) and sporulated immediately, before the loss of [PSI+ss] in the diploids could occur, the segregation was 2 Ura+ $G418^{res}$: 2 Ura− $G418^{sen}$ (29 tetrads).

Table 1. [PSI+ss] is more stable in *siw14*Δ than in the *siw14*Δ/+ host

Isolate no.	Ura+/total subclones	
	*siw14*Δ	*siw14*Δ/+
1	48/48	4/25
2	41/41	9/29
3	35/35	1/16
4	39/40	0/26
5	31/31	1/17
6	31/36	2/22
7	50/50	2/12
8	41/41	2/23

Ura+, guanidine-curable clones induced in strain 5255 (*siw14*Δ [PIN+]) were subcloned on YPAD medium or mated with strain 4729 (*SIW14* [psi−]) and then subcloned on YPAD medium. Subclones were tested for [PSI+] by replica-plating on −Ura plates.

These experiments show that *siw14*Δ does not simply affect the expression of the [PSI+] phenotype but determines the stability of prion propagation. The cosegregation of Ura+ and $G418^{res}$ also shows that it is indeed the *siw14*::*kanMX* allele that allows the propagation of [PSI+ss], and not some other incidental mutation in the strain.

Several [PSI+] variants (all guanidine-curable and mitotically stable) were isolated anew in 5255 (*siw14*Δ [PIN+] [psi−]) and cytoduced into isogenic WT and *siw14*Δ recipients (Table 2). Some variants were equally transmitted to both recipients, but the [PSI+ss] variants were better transmitted to the *siw14*Δ recipient. To confirm that cytoductants from *siw14*Δ [PSI+ss] into WT cells lost [PSI+ss], the Ura− cytoductants were used as donors to return the cytoplasm to a *siw14*Δ environment (strain 5255). These back cytoductants were uniformly Ura−, including experiments with three independent [PSI+ss]s (Table 3).

To further confirm that it is the *siw14*Δ mutation that allows propagation of [PSI+ss], we transformed *siw14*Δ [PSI+ss] strains with a *CEN* plasmid carrying *SIW14* driven by its own promoter and found curing by this plasmid (compared with the empty vector) (Table 4).

***siw14*Δ Does Not Affect Translation Termination Readthrough.** The above experiments prove that *SIW14* blocks propagation of [PSI+ss] variants. However, to determine whether Siw14p also affects translation termination readthrough, we used a dual luciferase plasmid with a 5′ *Renilla* luciferase gene separated by a UAA codon from an in-frame 3′ firefly luciferase gene (46). We

Table 2. Cytoduction of Siw14p-sensitive and -insensitive [PSI+] isolates

[PSI+] donor isolate no.	Recipient WT strain 5335		Recipient *siw14*Δ strain 5337		Stability of [PSI+] donor	
	[PSI+]	[psi−]	[PSI+]	[psi−]	[PSI+]	[psi−]
1	17	2	13	0	33	0
9	15	3	18	0	38	0
14*	1	27	16	7	67	0
21*	0	39	11	11	30	0
20*	0	31	15	10	32	0

A series of [PSI+]s (each guanidine curable) was generated in strain 5255 (*siw14* [PIN^+] [psi−]). Cytoductions were performed into isogenic WT strain 5335 and *siw14*Δ strain 5337 recipients, and the original [PSI+] clones were subcloned to determine their stability.
*Isolates 14, 20, and 21 are [PSI+ss].

Table 3. Back-cytoduction proves [PSI+ss] is lost in a WT strain

[PSI+ss] donor 1	WT recipient 1	*siw14*Δ recipient 2
	Cytoductants 1 = donor 2	Cytoductants 2
PB7 *siw14* [PSI+ss]	2 Ura+, 9 Ura−	All Ura− (>17)
PB14 *siw14* [PSI+ss]	10 Ura−	All Ura−
5355 *siw14* [PSI+ss]	15 Ura−	All Ura−
[psi−] control	20 Ura−	All Ura−

Recipient 1 is WT strain 5402, and recipient 2 is *siw14*Δ strain 5255, each isogenic with the donors but initially [psi−]. Ura− cytoductants of cytoduction 1 were used as donors in cytoduction 2.

examined two sets of isogenic *siw14*Δ [psi−] and WT [psi−] strains (Table 5). There were minor differences, but no consistent effect of *siw14*Δ on translational readthrough was observed.

Siw14p encodes a pyrophosphatase specific for 5PP-IP_5 (Fig. 1) (32). A *siw14*Δ strain has substantially elevated levels of 5PP-IP_5 and 1,5PP-IP_4 (32), suggesting that one or both of these compounds may have effects favorable to the propagation of some [PSI+] variants.

Arg82p Is Necessary for Most [PSI+] Variants. Arg82 is an IP multikinase converting IP_3 to IP_4 and then to IP_5 (47, 48). The Arg82p kinase activity is necessary for the synthesis of 5PP-IP_5 and all other inositol poly/pyrophosphates with more than three phosphates (47, 48). Our finding that Siw14p, which lowers the levels of certain inositol poly/pyrophosphates, antagonizes the propagation of certain [PSI+] variants suggests that other genetic modifications that lower the levels of these compounds may have a similar effect. We therefore tested whether *arg82*Δ has a similar effect. We prepared an *arg82*Δ *lys2* strain carrying a single-copy *LYS2* plasmid with *ARG82* under its own promoter (p1574) as well as p1520 carrying *ura3-14* and *GAL1-SUP35NM* on a *LEU2 CEN* plasmid for [PSI+] induction and detection.

We induced [PSI+] formation in this essentially WT strain (5478) by growth in galactose for 24 h and selected [PSI+] clones by plating on −Ura −Leu −Lys medium. Guanidine-curable clones were identified as [PSI+] (called "[PSI+1]," "[PSI+2]," and so forth), and the loss of the *ARG82* plasmid from such clones was then selected by plating on α-aminoadipate plates (49) or was screened for among colonies growing on rich medium by replica-plating to −Lys medium (Fig. 2*A*). For each of the 16 [PSI+] isolates tested, loss of p*LYS2-ARG82* resulted in all cells becoming Ura−, but nearly all clones retaining p*LYS2-ARG82* remained Ura+. Retransformation of the *arg82*Δ Ura− clones with p1574 (p*LYS2-ARG82*) resulted in most cells remaining Ura−. A minority of cells (13 of 64 for [PSI+3], 27 of 88 for [PSI+1]) became Ura+ again, indicating that loss of *ARG82* did not eliminate the prion completely in all cells. However, essentially all *arg82*Δ cells were Ura−. We suggest that the minority *arg82*Δ Ura− [PSI+] cells had seed number so low that there was no phenotype, while the majority of cells had completely lost [PSI+].

To test whether the *arg82*Δ mutation might directly affect translation terminator readthrough efficiency, we used the dual luciferase system mentioned above (Table 5). We found that *arg82*Δ resulted in a significant increase in readthrough efficiency in [psi−] strains, a result previously reported using a different tandem reporter plasmid (50). This result is the opposite of what would be expected if a direct effect on translation were to explain the Ura− phenotype produced from [PSI+] *arg82*Δ p*ARG82* cells on loss of p*ARG82*. We infer that most *arg82*Δ cells have become [psi−] and that the minority that are not [psi−] by the retransformation test have a very low seed number.

Similar experiments were carried out in the 779-6A background using the [PSI+]-suppressible *ade2-1* as the reporter, with similar results. The *arg82*Δ mutation and p*ARG82* (p1574) were

Table 4. Transforming *siw14Δ* [PSI+ss] with *SIW14* cures [PSI+ss]

	[PSI+ss] *siw14Δ* strain transformant phenotype: %Ura+ (total transformants)		
Plasmid	PB7	PB14	5355
Vector pH321	75 (57)	97 (331)	76 (238)
p*SIW14* p1569	20 (570)	36 (312)	13 (686)

introduced, [PSI+] clones were induced, and loss of p*ARG82* resulted in the loss of [PSI+] in each case (eight variants were tested).

The Kinase Activity of Arg82p Is Necessary for Propagation of [PSI+]. In addition to its inositol multikinase activity (47, 48), Arg82p is known to stabilize the essential transcription factor Mcm1p (51), facilitating mating, cell-cycle events, osmotolerance, and arginine metabolism (52, 53). The Mcm1p stabilization does not require the kinase activity, as inactivation of the kinase by the mutations D131A and K133A does not impair these activities (52, 54). To determine the role of the Arg82 kinase activity in [PSI+] propagation, we constructed an *arg82*Δ [PSI+] strain carrying p1574 (*CEN LYS2 ARG82*) as well as p1585 [*CEN HIS3 arg82*(D131A K133A)] and p1520 (*CEN LEU2 ura3-14 Gal1p-NM*). Upon loss of the *LYS2 ARG82* plasmid, only the kinase-defective Arg82p$^{D131A\ K133A}$ is available, and [PSI+] is uniformly lost (Fig. 2*B*). This shows that the IP multikinase activity of Arg82p is responsible for the propagation of the [PSI+] prion.

ARG82 is immediately adjacent to *SUP35* on chromosome IV with converging transcription and overlapping 3′ UTRs. One could suggest that the direct effect of *arg82*Δ on terminator readthrough (Table 5) and the loss of [PSI+] might both be a result of decreased *SUP35* expression. However, the fact that *ARG82* on a plasmid supports [PSI+] while the *arg82*(D131A K133A) mutant on a plasmid does not argues strongly against this interpretation. Moreover, as shown below, other mutants in the IP pathway in genes not located near *SUP35* also lose [PSI+].

[PSI+] Propagation in Inositol Poly/Pyrophosphate Mutants. To further narrow the range of possible IP species that may be involved in [PSI+] propagation, we used cytoduction to pass each of two Arg82p-dependent [PSI+] variants to an array of single and double mutants with altered IP metabolism (Fig. 1*A* and Table 6). The *arg82*Δ strains are deficient in all IPs above IP_3 but accumulate elevated levels of its substrate, IP_3 (48, 55). If IP_3 were an inhibitor of [PSI+] propagation, then *plc1*Δ strains, unable to make IP_3, should be able to propagate the prion (Fig. 1*A*). However, like *arg82*Δ mutants, *plc1*Δ strains could not propagate either [PSI+] variant (Table 6), showing that it is a product of Arg82 that is needed for [PSI+] rather than inhibition by its accumulated substrate.

[PSI+1] and [PSI+2] were efficiently transmitted by cytoduction to several single mutants in IP metabolism, including *vip1*Δ, *kcs1*Δ, *ipk1*Δ, *siw14*Δ, and *ddp1*Δ. However, *ipk1*Δ *kcs1*Δ or *kcs1*Δ *ddp1*Δ double mutants uniformly lost [PSI+] (Fig. 1*A* and Table 6). Mating the Ura− *ipk1*Δ *kcs1*Δ or *kcs1*Δ *ddp1*Δ double mutants with an isogenic WT strain produced all Ura− diploids except for a very rare Ura+ diploid. This shows that [PSI+] was indeed lost from the *ipk1*Δ *kcs1*Δ or *kcs1*Δ *ddp1*Δ double mutants and that the mutations did not simply affect the phenotype. Note that [PSI+] can propagate in the *vip1*Δ *kcs1*Δ double mutant, lacking all known inositol pyrophosphate-synthesizing enzymes. This indicates either that another inositol pyrophosphate-synthesizing enzyme exists or that some IP, presumably IP_6, made by Ipk1p, can help [PSI+] (*Discussion*).

In addition to acting on IP_3, Arg82p can phosphorylate phosphatidylinositol-4,5-diphosphate [$PI(4,5)P_2$] to form phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] (Fig. 1*A*) (56). If this were sufficient for prion propagation, then the double mutant *ipk1*Δ *kcs1*Δ would be able to propagate [PSI+] because neither protein is involved in making $PI(3,4,5)P_3$. However, the double mutant cannot propagate [PSI+]. The inability of the *plc1*Δ strain to propagate [PSI+] supports this conclusion as well, because this mutant should not be impaired in making $PI(3,4,5)P_3$.

Cytoduction of [PSI+1] or [PSI+2] into another *arg82*Δ p*ARG82* strain produced only Ura+ cytoductants that again lost [PSI+] on loss of p*ARG82* (Table 6). This important control shows that the [PSI+] variants had not changed since their initial isolation.

Overproduction of Siw14p Does Not Cure [PSI+]. The [PSI+] strain 779-6A (assaying [PSI+] using the suppressible allele *ade2-1*) was transformed with p1534 (*GAL1* promoter-*SIW14*) or the vector (pH773), and transformants were grown on galactose for 2 d and then plated on 1/2 yeast extract/peptone/dextrose (YPD) medium to detect [PSI+] loss by the red pigment that accumulates in unsuppressed *ade2* mutants. There was no increase in [psi−] clones. In another experiment, strain 74D-694 (*ade1-14* [PIN+] [psi−]) carrying pSL1066 (*CUP1* promoter, *SUP35NM*) and p1534 (*GAL1* promoter, *SIW14*) was grown with copper to induce [PSI+] appearance, and 12 variants were tested for increased loss of [PSI+] after growth in galactose for 3 d. None showed greater instability than with the glucose control. Thus, this overproduction of SIW14p does not cure [PSI+] variants isolated at normal levels of the protein.

[PSI+] Variants Independent of *ARG82*. Although the loss of the p*ARG82* from *arg82*Δ p*ARG82* [PSI+] Ura+ strains results in apparently uniformly Ura− cells, incubation for >1 wk results in growth of rare Ura+ clones. Each is guanidine-curable and produces frequent Ura− subclones in the absence of guanidine, suggesting that these are unstable, Arg82p-independent [PSI+] variants.

Is the Loss of [PSI+] in *arg82*Δ Cells a Result of Impairment of the Environmental Stress Response? Cells exposed to a variety of stresses, including high salt, oxidation, and heat shock, respond by shutting down translation and turning on stress-response genes. This response is controlled by inositol pyrophosphates, and in *arg82*Δ strains the environmental stress response (ESR) is not effective (57). To test whether this system is responsible for our observation of the involvement of IPs in [PSI+] prion

Table 5. Direct effects of *siw14*Δ and *arg82*Δ on translation termination efficiency

	Firefly luciferase/*Renilla* luciferase		
Genotype (strain)	Experiment 1	Experiment 2	Experiment 3
WT (4812)	0.0030		
*siw14*Δ (5255)	0.0040		
*siw14*Δ (5261)	0.0026		
WT (4813)	0.0010		
*siw14*Δ (5337)	0.0032		
WT (4812)	0.0053	0.0010	0.0012
*arg82*Δ (5408)	0.014	0.015	0.015
WT (4813)	0.015	0.0015	0.0019
*arg82*Δ (5477)	0.052	0.0020	0.044

Cells expressed *Renilla* luciferase upstream, separated by a UAA codon from firefly luciferase downstream from a single mRNA. The ratios show relative, not absolute, terminator readthrough rates because the enzyme activities are different. All strains tested were [psi−]. [PSI+] strains have values in the range 1.0–2.0.

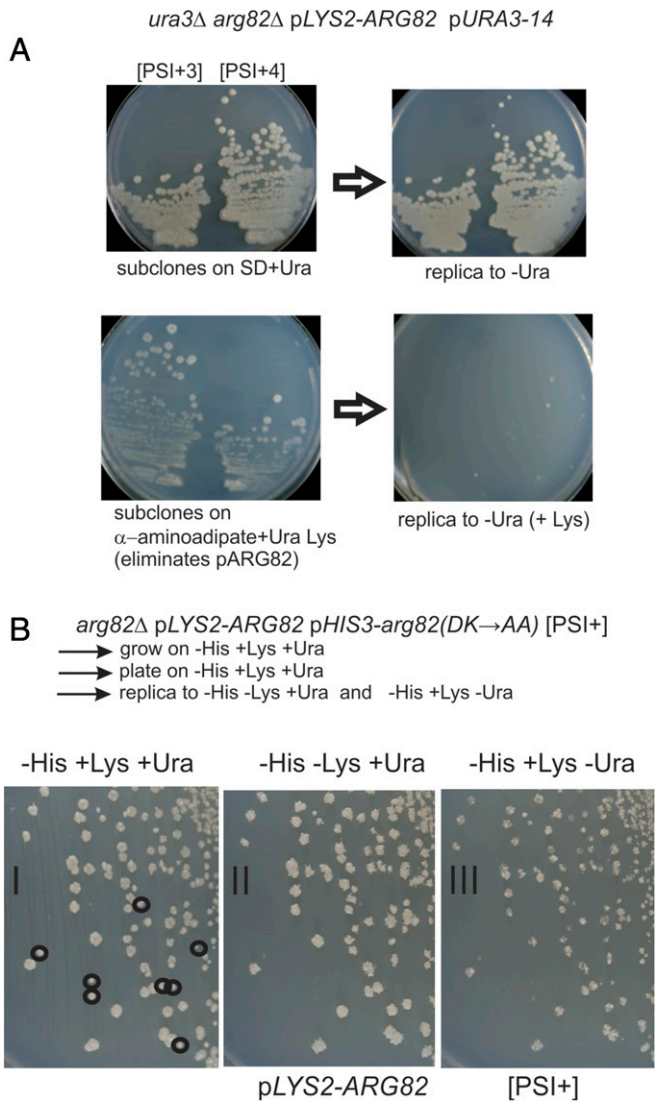

Fig. 2. (*A*) [PSI+] requires Arg82p for its propagation. [PSI+] cells with chromosomal *arg82*Δ and p*LYS2-ARG82* become Ura– ([psi–]) when loss of the plasmid is selected on α-aminoadipate medium (selects *lys2*–). (*B*) The kinase activity of Arg82p is required for propagation of [PSI+]. The circled colonies in the left panel have lost p*LYS2-ARG82* (i.e., fail to grow on –Lys in the middle panel) but retain p*HIS3-arg82(D131A K133A)* expressing the kinase-dead Arg82p. All such colonies are Ura– (i.e., fail to grow on –Ura in the right panel), showing that they cannot maintain [PSI$^+$].

propagation, we tested cytoduction of two *ARG82*-requiring [PSI+] variants to strains carrying *kcs1*Δ *vip1*Δ. In *kcs1*Δ *vip1*Δ strains inositol pyrophosphate synthesis is blocked, and there is little or no ESR (57), but this mutant combination has no effect on [PSI+] propagation (Table 6). In addition, in our experiments, we do not subject the cells to any of the known inducers of the ESR. Thus [PSI+] propagation is not dependent on the ESR.

[URE3] Is Not Lost from *arg82*Δ Strains. *DAL5* is strongly repressed by active Ure2p in medium with a good nitrogen source, such as ammonia (58). Placing the *ADE2* gene under the *DAL5* promoter enables assay of the loss of Ure2p activity in a [URE3] strain as an Ade+ phenotype (11, 59). Strain BY241 (*DAL5:ADE2*) was made *arg82*Δ *lys2*, and p1330 (*GAL-URE2N*) and p1574 (p*LYS2 ARG82*) were introduced. [URE3] prion formation was induced by overproduction of the Ure2p prion domain (Ure2N). Sixteen Ade+ clones were tested for curing on 5 mM guanidine, and 15 were found to be curable. These 15 [URE3] isolates were grown to single colonies on either SD medium without lysine, to ensure they remain *ARG82*$^+$, or on α-aminoadipate medium to select for loss of the *ARG82* plasmid. For these 15 [URE3] isolates, there was no increase in loss of the prion following loss of *ARG82*.

Relation of Inositol Poly/Pyrophosphate Effects on [PSI+] to the Hsp104 [PSI+]-Curing Activity. The disaggregating chaperone Hsp104, when overproduced, cures [PSI+] (25, 26), but Hsp104^{T160M} lacks this activity (28). Normal levels of Hsp104 cure more than half of [PSI+] variants arising in the *hsp104*^{T160M} mutant, showing that this constitutes an antiprion system (29). Two [PSI+] variants dependent on *ARG82* for their propagation were cytoduced into isogenic *arg82*Δ *hsp104*^{T160M} p*LYS2-ARG82* and *arg82*Δ *HSP104* p*LYS2-ARG82* hosts. Loss of the p*LYS2-ARG82* plasmid resulted in the uniform loss of each [PSI+] variant in both hosts (Table 7). This shows that the inositol poly/pyrophosphate requirement for [PSI+] does not operate through Hsp104's [PSI+]-curing activity.

Possible Targets of IP Action in Controlling [PSI+]. Among the IP_6/5PP-IP_5–binding proteins identified by Wu et al. were two chaperones, Sse1p and Hsp26p (60). Sse1p overproduction or deficiency cures [URE3], and *sse1*Δ strains lose a weak [PSI+] (61). In addition, overproduction of Sse1p stimulates [PSI+] generation, and Sse1p deficiency deters the formation of this prion (62). Deficiency of Hsp26p destabilizes [URE3] (31). Hsp42p is necessary for Btn2p overproduction curing of [URE3-1], and overproduction of Hsp42p itself can cure [URE3-1] (31). Thus, Hsp42p is also a candidate for involvement in [PSI+] curing. One group reports that overproduction of Hsp26 or Hsp42 cures [PSI+] (63), but we did not find that either cures [PSI+] (31). In this work, we also found that CEN plasmids expressing Hsp26 or Hsp42 from the very strong *GPD1* promoter failed to produce curing of [PSI+1] or [PSI+2]. We also find here that Sse1p overproduction sufficient to cure [URE3-1] did not prevent the loss of [PSI+1] or [PSI+2] on the loss of Arg82p. Likewise, expression of Hsp26 from a high-copy plasmid (p1606) did not affect the loss of these [PSI+] variants in *arg82* cells. Neither *hsp26*Δ nor *hsp42*Δ results in loss of [PSI+1] or [PSI+2] from an otherwise WT host, as shown by efficient cytoduction of either variant from strains 5548 or 5549 into the respective knockout mutant (Table 8). Further work will be required to identify the target of IP action affecting prion propagation.

Discussion

Our search for antiprion systems has found that elevated inositol poly/pyrophosphates in the *siw14*Δ mutant allows propagation of many [PSI+] variants that cannot propagate in a WT strain. In this sense, Siw14p is an antiprion factor. Our search method has the virtue of detecting prion curing in a WT strain not overexpressing or deficient for any proteins. The prion variants arose in a mutant, but probably not because of the mutation, as prion formation occurs readily in the absence of any other proteins in vitro (9–11, 64) and probably in vivo. We also find that elimination of most of the inositol poly/pyrophosphates by an *arg82*Δ mutation results in the loss of almost all [PSI+] variants.

What Inositol Pyro-/Polyphosphates Allow [PSI+] to Propagate? Because *arg82*Δ cells lose [PSI+], the IPs necessary for [PSI+] must be downstream of Arg82's kinase steps, unless IP_3, the substrate of Arg82, is inhibiting [PSI+] propagation. However, Plc1p, which produces IP_3, is also necessary for [PSI+], indicating that [PSI+] is not lost from inhibition by IP_3. The loss of [PSI+] in *plc1*Δ strains also argues that 1-phosphatidylinositol-3,4,5 phosphate, also produced by Arg82p, is not sufficient to support [PSI+] (Fig. 1*A*).

Although the only known effect of *siw14*Δ on the IPs is elevation of 5PP-IP_5 (and possibly 1,5PP-IP_4 or 5PP-IP_4) (32), both *kcs1*Δ and *kcs1*Δ *vip1*Δ mutants can propagate [PSI+], suggesting

Table 6. Propagation of [PSI+] in IP mutants

Donor *arg82Δ* p*ARG82* [PSI+x]	Recipient strain no.	% Ura+ cytoductants (total)	Donor *arg82Δ* p*ARG82* [PSI+x]	Recipient strain no.	% Ura+ cytoductants (total)
[PSI+1]	WT 4812	100 (19)	[PSI+1]	*plc1Δ* 5526	0 (10)
[PSI+2]		100 (6)	[PSI+2]		0 (14)
[PSI+1]	WT 4445	76 (25)	[PSI+4]	*kcs1Δ ipk1Δ*	0 (31)
[PSI+2]		94 (33)	[PSI+6]	5538	0 (21)
[PSI+1]	WT 5506	100 (13)	[PSI+1]	*kcs1Δ ipk1Δ*	0 (14)
[PSI+1]	*gal7Δ* 4866	100 (19)	[PSI+2]	5536	0 (5)
[PSI+2]		94 (17)	[PSI+1]	*kcs1Δ ipk1Δ*	0 (15)
[PSI+1]	*siw14Δ* 5498	94 (17)	[PSI+2]	5538	0 (16)
[PSI+2]		100 (30)	[PSI+1]	*kcs1Δ ddp1Δ*	0 (10)
[PSI+1]	*siw14Δ* 5505	92 (26)	[PSI+2]	5525	0 (8)
[PSI+2]		88 (17)	[PSI+4]	*kcs1Δ ddp1Δ*	0 (36)
[PSI+1]	*ipk1Δ* 5503	95 (19)	[PSI+6]	5525	0 (17)
[PSI+2]		96 (26)	[PSI+1]	*kcs1Δ vip1Δ*	88 (24)
[PSI+1]	*ipk1Δ* 5492	89 (28)	[PSI+2]	5440	83 (30)
[PSI+2]		88 (8)	[PSI+1]	*siw14Δ vip1Δ*	98 (85)
[PSI+1]	*kcs1Δ* 5502	100 (27)	[PSI+2]	5522	100 (68)
[PSI+2]		96 (25)	[PSI+1]	*siw14Δ kcs1Δ*	100 (20)
[PSI+1]	*kcs1Δ* 5495	100 (15)	[PSI+2]	5497	95 (38)
[PSI+2]		100% (19)	[PSI+1]	*siw14Δ ddp1Δ*	100 (20)
[PSI+1]	*vip1Δ* 5504	100% (22)	[PSI+2]	5457	100 (10)
[PSI+2]		100% (43)	[PSI+1]	*arg82Δ* p*ARG82*	100 (15)
[PSI+1]	*vip1Δ* 5496	100% (33)	[PSI+2]	5499	100 (20)
[PSI+2]		87% (30)	[PSI+1]	*ipk1Δ vip1Δ*	100 (29)
[PSI+1]	*ddp1Δ* 5494	100% (12)	[PSI+2]	5197-2D	100 (32)
[PSI+2]		100% (16)	[PSI+1]	*ipk1Δ vip1Δ*	100 (30)
			[PSI+2]	5197-5B	97 (41)

Each donor was 5478 *kar1Δ15* carrying one of four *ARG82*-dependent [PSI+] variants. As a control, the cytoductants into 5499 (*arg82Δ* p*ARG82*) were [PSI+] but became [psi−] on the loss of p*ARG82*.

that inositol pyrophosphates are not the only molecules capable of supporting [PSI+]. The failure of *kcs1Δ ipk1Δ* double mutants to propagate [PSI+] indicates that IP_6 can also fulfill the [PSI+]-promoting role. IP_6 is the only species missing in the *kcs1Δ ipk1Δ* double mutants that is present in the *kcs1Δ vip1Δ* strains. However, *ipk1Δ* single mutants propagate [PSI+] well, so IP_6 is not the only species capable of supporting [PSI+]. These results suggest that 5PP-IP_4 can help [PSI+]. If 5PP-I-1,4P (5PP-IP_2), another product of Kcs1p action, were sufficient to help [PSI+], then the *arg82Δ* strain would not lose [PSI+]. Assuming the current model of inositol poly/pyrophosphate synthesis is correct, our results prove that IP_6 and 5PP-IP_4 are each sufficient to support [PSI+] propagation and that 5PP-IP_5 and 1,5PP-IP_4 are likely also capable of enabling [PSI+] propagation.

The above inferences do not provide an explanation of the loss of [PSI+] in the *kcs1Δ ddp1Δ* double mutants but not in either single mutant. If 1PP-IP_5 were an inhibitor of [PSI+], its elevation in a *ddp1Δ* mutant, coupled with a loss of the [PSI+]-helping 5PP-IP_5, would explain this result. However, expression of *VIP1* from a *GAL1* promoter in a *kcs1Δ* [PSI+] strain did not produce increased loss of the prion.

What is the Target of Inositol Poly/Pyrophosphates in Enabling [PSI+] Propagation? Three components previously known to be involved in mRNA export from the nucleus, Dbp5p, Gle1p, and IP6, are now known to be necessary for efficient translation termination (50, 65, 66). IP_6 (inositol hexakisphosphate) binds to Gle1p, and both Gle1p and Dbp5p bind to Sup45p, the partner of Sup35p in the translation termination complex. Thus, IP-deficient *ipk1* mutants show inefficient translation termination, the opposite of the increased translation termination efficiency we see in *ipk1Δ kcs1Δ* strains due to the loss of [PSI+]. Thus, we cannot explain our results as simply the effect of IP_6 binding to Gle1. However, further work will be needed to determine whether there is some indirect relation of these two inositol poly/pyrophosphate effects.

Recently, Wu et al. prepared affinity reagents to capture proteins binding to IP_6 or 5PP-IP_5 and isolated proteins from extracts of *S. cerevisiae* that were specifically bound (60). Among those identified were Sse1p, Hsp26p, and Ssb1,2p. Remarkably, most proteins isolated had similar affinity for the IP_6 and the 5PP-IP_5 affinity substrates (60), similar to the apparent ability, in our experiments, for either IP_6 or a Kcs1p product (presumably 5PP-IP_4 or 5PP-IP_5) to support [PSI+] propagation. As discussed above, Ssbs lower the frequency of [PSI+] arising but do not cure [PSI+] variants produced in their absence (24). Sse1p is known to be critical for propagation of the [URE3] prion, with either overproduction or deficiency resulting in curing (61). It was found that a weak [PSI+] prion was lost from an *sse1Δ* strain, but a strong [PSI+] was maintained with a weakened phenotype (61). While overproduction of Sse1p enhanced [PSI+] generation, deficiency severely restricted it with a limited range of variants found (62). This makes Sse1p a candidate for mediating IP effects on [PSI+]. However, while all of the 24 [PSI+] tested here (including many strong [PSI+]) were lost from an *arg82Δ*, a strong [PSI+] was not lost from an Sse1Δ strain (61).

Hsp26p and Hsp42p are small heat-shock proteins, oligomeric inhibitors of protein aggregation with α-crystallin domains characteristic of this group (67). Hsp26 and Hsp42 are reported to block Sup35p amyloid formation and propagation in vitro (63). However, we find that neither deficiency nor overproduction of either of these proteins affects the propagation of either of two Arg82p-requiring [PSI+] variants in a WT strain. Further work will be needed to detail the mechanism of inositol poly/pyrophosphates on prion propagation. The IP may be stimulating, or inhibiting, or change the specificity of one or more of the components involved in prion propagation. IPs and pyrophosphates affect a wide array of

Table 7. Loss of [PSI+] from *arg82Δ* cells does not require the Hsp104 antiprion activity

Strain	Genotype	Lys^- clones % Ade^+ (no.)	Lys^+ clones % Ade^+ (>50)
5565	*arg82Δ* p*LYS2-ARG82* [PSI+1] *HSP104*	0 (25)	100
5571	*arg82Δ* p*LYS2-ARG82* [PSI+1] $hsp104^{T160M}$	0 (18)	100
5565	*arg82Δ* p*LYS2-ARG82* [PSI+2] *HSP104*	0 (18)	100
5571	*arg82Δ* p*LYS2-ARG82* [PSI+2] $hsp104^{T160M}$	0 (12)	100

Strains 5565 (*HSP104*) and 5571 ($hsp104^{T160M}$) were used as cytoduction recipients for [PSI+1] and [PSI+2] from strains 5552 and 5553. All cytoductants were Ade^+ ([PSI^+]). Single cytoductants (two for each genotype) were subcloned in the presence of lysine and adenine to allow plasmid loss. Clones were replica-plated to Lys^- and Ade^- plates. The $hsp104^{T160M}$ allele, which eliminates the Hsp104 antiprion activity, does not affect the requirement of [PSI+1] or [PSI+2] for *ARG82*.

cellular processes, most of them seemingly not likely to impinge on the process of prion propagation. In most cases, the direct target of the inositol poly/pyrophosphate is not known. Further work will be required to trace the pathway of prion control by these signal transducers. The known parallel of IP pathways between yeast and humans (33) and our finding of a previously unrecognized mode of control of prion propagation open possible avenues for control of a range of amyloid-based diseases.

Methods

Media and Strains. Rich medium (YPAD), minimal medium (SD), and sporulation medium were as described (68). Low-adenine rich medium (1/2 YPD) is 0.5% yeast extract, 2% peptone, 2% dextrose, and 2% agar. Most strains used were isogenic to BY4741 (69). Most knockouts were made by PCR amplification of the *gene::kanMX* module from the knockout collection including 200 bp on each side, transformation selecting for G418 resistance, and confirmation by PCR with internal primers (kanB and kanC inside *kanMX* or primers inside the normal gene) and primers 300 bp 5′ or 3′ to the ORF being disrupted. The presence of the disruption and the absence of the normal gene was confirmed in each case. Double-mutant strains were constructed by meiotic crosses of two (isogenic) knockout bank strains and scoring of the segregants by PCR. Strains with the *kar1Δ15* allele (70) were made as described (71) (see Table S1).

In most experiments [PSI+] is measured using the *ura3-14* allele, a nonsense allele suppressible by the partial deficiency of the translation termination factor Sup35p resulting from its being largely sequestered in amyloid filaments. In some experiments (e.g., Table 7) [PSI+] is measured using suppression of *ade2-1* with *SUQ5*. [URE3] was assayed using a *DAL5* promoter-*ADE2* fusion since normal Ure2p represses *DAL5* transcription (58).

p*GAL1-SUP35NM-URA3-14*. We combined the *URA3-14* plasmid useful for detecting [PSI+] (37) with the *GAL1* promoter/*SUP35NM*/*ADH1* terminator from pHK006 (72). The *GAL1* promoter/Sup35NM/*ADH1* terminator from pHK006 was amplified using primers with 40-bp ends that were homologous to regions of pLEU2Ura3-14 surrounding the NaeI restriction site, between the PGK terminator of *URA3-14* and the leucine tRNA gene adjacent to *LEU2*. pLEU2Ura3-14 was cut with NaeI and transformed into yeast strain YB4741 (*MATa his3 met5 leu2 ura3*) with the amplified purified Gal-SUP35NM fragment, selecting Leu+. Colony PCR identified clones with the desired homologous recombination event. The plasmid (p1520) was isolated and sequenced (see *Supporting Information*).

Table 8. Hsp26 and Hsp42 are dispensable for [PSI+] propagation

Donor *arg82Δ* p*ARG82* [PSI+x]	Recipient strain no. (ρ^o p1520 pRS313)	%Ura+ cytoductants (total)
5548 [PSI+1]	WT, 4812	88 (25)
5549 [PSI+2]	WT, 4812	86 (14)
5548 [PSI+1]	*ipk1Δ kcs1Δ*, 5536	0 (15)
5549 [PSI+2]	*ipk1Δ kcs1Δ*, 5536	0 (22)
5548 [PSI+1]	*hsp26Δ*, 5558	98 (40)
5549 [PSI+2]	*hsp26Δ*, 5558	100 (23)
5548 [PSI+1]	*hsp42Δ*, 5559	100 (25)
5549 [PSI+2]	*hsp42Δ*, 5559	90 (21)

Cytoduction donors were ρ^+, and recipients were ρ^o. Cytoductants were identified as those with recipient nuclear genotype and donor cytoplasm (ρ^+). The recipients carried p1520 bearing the *ura3-14* allele, enabling scoring of [PSI+] as Ura+.

p*SIW14* (p1569). *SIW14* and 500 bp of the upstream genomic sequence were amplified with oligos 978 and 979 including 5′ BamHI and 3′ HindIII sites; the product cut with these enzymes was inserted into pRS315 cut with the same enzymes. The *SIW14* gene was excised with the same enzymes and ligated to pH321 (pRS313 from which the HindIII and NheI sites were removed from the *HIS3* gene by site-directed mutagenesis), forming p1569.

p*LYS2-ARG82* (p1574). *ARG82*, including 408 bp of its 5′ upstream sequence, including all but 68 bp of the region between *ARG82* and the adjoining gene (*HMO1*), was amplified by PCR using oligos 1092 and 1093, cut with ApaI (a site present in the genome 408 bp upstream of the ORF) and SacI, and inserted into pRS317 (*CEN LYS2*) cut with the same enzymes, forming p1574.

Isolation of Antiprion Mutants. Pools of the *S. cerevisiae MATa* knockout bank (36) were made and transformed with p1520 carrying *GAL1-SUP35NM* and *ura3-14*, the latter suppressible by [PSI+] (Fig. 1*B*) (37). Thousands of transformant colonies from each pool were pooled, and an aliquot was grown in SGal medium (identical to SD medium but with galactose in place of dextrose) supplemented with uracil, histidine, and methionine for 24 h at 30 °C. Dilutions were plated on SD medium supplemented with histidine and methionine (i.e., –Ura) and grown for 6 d at 30 °C. These plates were replica-plated to YPAD with a seeded lawn of the isogenic WT *MATα* strain 4729. The mating on YPAD was allowed to proceed for 18 h, and then the plate was replica-plated to SD+His+Ura medium to confirm that mating had occurred and to SD+His medium to select [PSI+] diploids. Clones that formed diploids on SD+His+Ura but not on SD+His medium were candidates for antiprion mutants.

To confirm that candidates were [PSI+], each was streaked for single colonies on 1/2 YPD medium and on 1/2 YPD medium containing 5 mM guanidine hydrochloride. Colonies were replica-plated to SD+His+Met medium (i.e., –Ura). Only candidates becoming Ura– following exposure to guanidine but remaining Ura+ in its absence were examined further. Mating with WT strain 4729 was repeated to confirm that diploids were Ura–. DNA from candidates was extracted using the YeaStar Genomic DNA Kit (Zymo Research), and the gene-specific barcode sequences embedded in each KanMX-knockout cassette were amplified using primers U1 (73) and KanB.

Cytoduction. Recipients were made ρ^o by growth on rich medium containing 30 μg/mL of ethidium bromide, and donors were ρ^+. In some experiments, recipients also carried pRS313, a *CEN HIS3* vector, to enable selecting against donor cells after the mating period. Mating mixtures were incubated for 7 h on YPAD medium and then were streaked for single colonies on media selecting against the donor. Clones were replica-plated to medium allowing only diploids to grow, to glycerol medium (to check for transfer of cytoplasm), and to –Ura or –Ade medium as appropriate for transfer and propagation of the prion.

Using Dual Luciferase Vectors to Measure Translation Termination Efficiency. We used a plasmid (pSC5) constructed by Harger and Dinman in which the upstream *Renilla* luciferase was fused in frame to the firefly luciferase but with a UAA stop codon at the sixth codon of the downstream firefly luciferase section (46). The two luciferase activities were separately assayed in the same preparation using the Dual-Glo Luciferase Assay System (Promega) with a Berthold luminometer (Titertek Berthold). Cells were grown to late log phase in media selective for retention of pSC5, washed with water, and lysed with the Dual-Glo Luciferase Reagent, and firefly luciferase activity was measured. Then the Stop & Glo reagent was added, which inhibits the firefly

luciferase ~10,000-fold but allows the *Renilla* luciferase reaction to proceed. The two luciferases are expressed as a fusion protein from the same mRNA, providing an internal control for mRNA amounts.

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