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ORIGINAL ARTICLE

Total propagation of yeast prion conformers in *ssz1Δ upf1Δ* Hsp104^{T160M} triple mutants

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Abstract

It was reported that yeast proteins Ssz1 and Upf1 can cure certain [*PSI*$^+$] variants in wild-type cells and there is a special class of variants whose propagation requires the triple mutation of *ssz1Δ upf1Δ* Hsp104^{T160M}. Attempts to isolate variants with the exact properties from the 74-D694 strain (and tested there) are not yet successful. The effort nevertheless leads to an alternative analysis about how *ssz1Δ* and *upf1Δ* mutations can help prion propagation. The cellular propagation of the yeast prion [*PSI*$^+$] requires appropriate activities of the Hsp104 disaggregase. Many [*PSI*$^+$] variants isolated in wild-type strains cannot propagate in cells expressing Hsp104^{T160M}, which has weaker activities. Yet another group of [*PSI*$^+$] variants shows the opposite, propagating well with Hsp104^{T160M} but is eliminated by the wild-type protein. Deletion of *SSZ1* and *UPF1* genes in Hsp104^{T160M} cells generates a just-right environment that supports the propagation of both types of [*PSI*$^+$] variants. The pro-prion effect is not due to the removal of active curing by Ssz1 or Upf1–such curing activity is not observed for the variants. Rather, the double deletion causes a cellular response, which enables more efficient fragmentation of prion fibers, thus remedying the weak activity of Hsp104^{T160M}. The "Goldilocks" conditioning seems also applicable to other yeast prions. Two [*PIN*$^+$] variants that propagate well with wild-type Hsp104 but poorly with Hsp104$^{\Delta N}$, lacking residues (2-147), can however thrive with the latter if Ssz1 and Upf1 are also deleted from the cell. In this case, the double deletion results in higher Hsp104$^{\Delta N}$ expression, leading to improved generation of prion seeds for robust propagation.

Keywords [*PSI*$^+$] · Prion · Variants · Amyloid · *UPF1* · *SSZ1* · *Saccharomyces cerevisiae*

Introduction

[*PSI*$^+$] variants are different amyloid conformers of the yeast Sup35 protein (Wickner 2016; Cox 1965). [*PSI*$^+$] amyloid captures free Sup35 in the cell and acts as templates to refold and assimilate it. The Hsp104 disaggregase cuts long amyloid fibers into pieces, ensuring that daughter cells can get sufficient seeds to continue the replication cycle (Chernoff et al. 1995; Kryndushkin et al. 2003; Kushnirov and Ter-Avanesyan 1998). Inadequate severing by the disaggregase thus cause a cell lineage to lose [*PSI*$^+$]. Excessive Hsp104 activities also cure [*PSI*$^+$] (Chernoff et al. 1995). There are different theories about the exact molecular mechanism for this (Park et al. 2014; Ness et al. 2017; Winkler et al. 2012; Cox and Tuite 2018) but several lines of evidence indicate that excess Hsp104 cures [*PSI*$^+$] by simply dissolving prion seeds in the cell (Park et al. 2014; Zhao et al. 2017; Huang et al. 2020).

Sup35 is a subunit of the yeast translation termination complex. [*PSI*$^+$] aggregates sequester free Sup35, lower its effective cellular concentration, and thus cause stop codon readthrough, which is detected genetically by the suppression of nonsense mutations (Cox 1965). Alternatively, [*PSI*$^+$] particles can be observed in vivo by labeling with Sup35-GFP fusion constructs (Patino et al. 1996; King 2001).

Gorkovskiy et al. (2017) observed that many [*PSI*$^+$] isolates obtained from yeast expressing Hsp104^{T160M} are cured when re-propagated in wild-type cells. We subsequently characterized several of them. The variants turn out to be

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slightly mutable, occasionally mis-seeding a different conformation that can survive the wild-type cell (Huang et al. 2020). However, some of the most studied [*PSI*$^+$] variants isolated from wild-type strains cannot be maintained in cells expressing Hsp104^{T160M} (designated "T160M cells", hereinafter). The curing can nevertheless be mitigated by overexpression of the mutant Hsp104. We further determined that the Hsp104^{T160M}-compatible variants have lower thermal stability than the classical ones. These results suggest that Hsp104^{T160M} is a weaker disaggregase. At normal cellular expression level, its collective activity is too low to generate enough prion seeds for the classical variants but sufficient for the less stable ones.

In a series of publications, Son and Wickner (2018, 2020, 2022) reported several novel classes of [*PSI*$^+$] variants which arise in yeast background without *UPF1*, *SSB1/2* or *SSZ1* (designated *upf1*Δ, *ssb1/2*Δ, and *ssz1*Δ) but are cured when the gene functions are restored. The proteins perform important functions in cell quality control, helping to make sure that polypeptides emerging from the ribosome are correct or can fold correctly. Upf1 is a helicase involved in nonsense mediated RNA degradation. It interacts with Sup35 in vivo and the interaction prevents Sup35 amyloid formation in vitro (Czaplinski et al. 1998; Son and Wickner 2018). [*PSI*$^+$] colonies were reported to arise 10 times more frequently in *upf1*Δ cells than the wild-type counterpart (Son and Wickner 2018). Ssb1/2 and Ssz1 are molecular chaperones associated with the ribosome. Deleting *SSB1/2* increases the frequency of [*PSI*$^+$] colonies arising (Chernoff et al. 1999; Son and Wickner 2018). Excess Ssb1/2 causes mitotic instability of some [*PSI*$^+$] variants (Kushnirov et al. 2000; Chacinska et al. 2001; Allen et al. 2005; Chernoff et al. 1999). Phenotypes for *ssz1*Δ are more complex. Deletion of *SSZ1* increases [*PSI*$^+$] induction but also destabilizes the propagation of some [*PSI*$^+$] variants (Kiktev et al. 2015; Amor et al. 2015). It was proposed that the deletion increases the chance for nascent polypeptides to misfold, including that of Sup35, thus enhancing [*PSI*$^+$] formation. The deletion triggers a chain of events. Ribosome-associated Ssb1/2 are released to the cytosol, disturbing the interaction between the homologous Ssa1 protein and Hsp104, consequently reducing the severance of prion fibers (or more probably enhancing the trimming and dissolution of prion particles; see discussion), which in turn leads to [*PSI*$^+$] instability (Kiktev et al. 2015). Independent of [*PSI*$^+$], *UPF1*, *SSB1/2* or *SSZ1* deletion also has impact on translation accuracy, causing mild increase in nonsense readthrough (Rakwalska and Rospert 2004; Moderazo et al. 2000).

We have previously compiled a list of 28 [*PSI*$^+$] variants and are keen to make the collection more complete (Huang and King 2020). Attempts were thus made to induce and characterize the novel variants in the 74-D694 strain. Unexpectedly, I did not yet find [*PSI*$^+$] variants that were cured by Ssb1/2, Ssz1, or Upf1 when induced and propagated in 74-D694. Instead, I observed that deletion of *SSZ1* and *UPF1* allowed T160M cells to propagate otherwise unsupported [*PSI*$^+$] variants from the wild-type cell without sacrificing the existing support for weaker variants.

Materials and methods

Yeast methods

All yeast experiments were performed with the 74-D694 genetic background (*ade1-14*(*UGA*) *ura3-52 leu2-3*,*112 trp1-289 his3-*Δ*200*; Chernoff et al. 1995). Cultures were handled according to Sherman (1991). ClonNAT (nourseothricin), G418 (geneticin), hygromycin B, cycloheximide, and 5-fluoroorotic acid (5-FOA) were used at the concentration of 100, 200, 300, 10, and 1000 mg/L, respectively. Centromere-based plasmids, YCp33(*URA3*) and YCp111(*LEU2*), and 2μ-based YEp195(*URA3*) were used as yeast vectors (Gietz and Sugino 1988).

Hsp104 mutations

T160M and ΔN strains were constructed by a two-step process. (1) The *HSP104* gene sequence was first interrupted with a *URA3* marker. A PCR product containing the coding sequence of Hsp104(41–56), followed by the marker and then the sequence of Hsp104(361–375) was integrated into the locus by homologous recombination. (2) PCR products containing T160M and ΔN sequences were introduced into cells to replace the *URA3* marker. Successful integration resulted in *ura3*$^-$ colonies, which were counter-selected on 5-FOA plates. For T160M, a PCR product encoding Hsp104(1-515)(T160M) was generated by the mega-primer method (Sarkar and Sommer 1990), which enabled seamless introduction of the T160M point mutation to *HSP104*. For ΔN, a plasmid carrying the sequence of *HSP104*$^{\Delta N}$ was first constructed, including a native 438-bp 5'-UTR, followed by a *Bam*HI restriction site, the initiation codon, the Hsp104(148–908) sequence plus the stop codon, and a 281-bp 3'-UTR. The plasmid was used as the template to obtain a PCR product spanning from the 5'-UTR to residue 515 of Hsp104. After genome integration, the *HSP104* gene lost the sequence for amino-acid residues 2-147, but gained an extra *Bam*HI site between the promoter and the start codon. Mutant *HSP104* alleles were confirmed by sequencing longer genomic PCR fragments which encompassed sequences used for homologous recombination.

Deletion strains

Dominant selection marker *Kan*MX and *Nat*MX4, conferring G418 and clonNAT resistance, respectively, was used to replace *SSB1*, *SSB2*, *SSZ1*, and *UPF1* genes by homologous recombination. DNA fragments containing the sequence of a dominant marker flanked by the first and the last 15 codons of target genes were obtained by PCR with appropriately designed oligos, using plasmids pUG6 (for G418; Goldstein and McCusker 1999) and pAG25 (clonNAT; Güldener et al. 1996) as the template. Drug resistant transformants were selected on YPD plates with G418 or clonNAT and were confirmed to have correct gene replacement by genomic PCR. Positive colonies were further streak-purified on selection plates and rechecked for gene deletions. Single deletion strains were crossed with each other to isolate spores with double deletions.

[*PSI*$^+$] induction

The procedure for [*PSI*$^+$] induction was described previously (Huang et al. 2020). Briefly, plasmids YEp195-Cup1-SupF (*URA3*), expressing the full-length Sup35 via a copper-inducible *Cup1* promoter, and YCp111-KanMX (*LEU2*), conferring G418 resistance to facilitate variant typing later, were introduced into yeast strains harboring the High [*PIN*$^+$] variant, which can propagate with both Hsp104^{WT} and Hsp104^{T160M}. Single colonies were grown in synthetic media without uracil and leucine (SC-UL). $CuSO_4$ was added to a final concentration of 50 µM to maximize Sup35 expression. Cultures were grown at 30 °C for 2 to 5 days to reach saturation. Aliquots were streaked on YPD + G418 or SC plates without adenine and leucine. For [*PSI*$^+$] selection on YPD + G418, plates were incubated at room temperature for about 10 days to allow colony color to fully develop. Non-red ([*PSI*$^+$]) colonies were picked without bias and transferred onto fresh plates in a grid pattern. For selection on SC-Ade, Leu, plates were incubated at room temperature for more than 3 weeks, and single colonies were randomly picked regardless of color and gridded onto fresh YPD + G418 plates. In both cases, colonies were replica-plated onto 5-FOA plates without leucine to lose YEp195-Cup1-SupF but keep YCp111-KanMX. They were replica plated to YPD + G418 again and were ready for variant type determination.

For [*PSI*$^+$] induction with p1520(*LEU2*), yeast colonies transformed with the plasmid were grown in liquid media containing 2% (g/ml) galactose and 1% raffinose to overexpress Sup35(1-253). After 4 days at 30 °C, aliquots of cultures were streaked on synthetic plates containing glucose but lacking uracil and leucine (SC-Leu, Ura). The plates were incubated at room temperature for 5 or 14 days (5 days for *upf1*Δ and Hsp104^{T160M}*upf1*Δ *ssz1*Δ strains, and 14 days for the *ssz1*Δ strain; *ura3-14* transcripts are more stable with *upf1*Δ). Colonies were randomly picked, regardless of the size, and placed onto fresh SC-Leu master plates in a grid pattern. Tiny colonies were not overlooked. To check if [*PSI*$^+$] was cured by excess Ssz1 and Upf1, the colonies were crossed with wild-type cells transformed with YEp-Hyg-Cup1-Ssz1 or YEp-Hyg-Cup1-Upf1, containing a 500-bp *CUP1* promoter, the hphMX4 marker, conferring hygromycin B resistance, and the Ssz1 or Upf1 coding sequence, followed by a 247-bp transcription terminator of *SUP35*. Diploids were selected on YPD plates containing G418 and hygromycin B. The 500-bp *CUP1* promoter is constitutively active in 74-D694.

To isolate spontaneously formed [*PSI*$^+$], yeast colonies carrying p1520 were grown in liquid media containing 2% (g/ml) glucose. After 2 days at 30 °C, aliquots of cultures were streaked on synthetic plates containing glucose but lacking uracil and leucine (SC-Leu, Ura). The plates were incubated at room temperature for 7 to 10 days. Small uracil prototrophic colonies were picked under a dissection microscope, transferred onto YPD + G418 plates to grow for 4 days at 30 °C, and then typed for [*PSI*$^+$] variation. [*psi*$^-$] colonies were identified by their red colony color and confirmed by the total and near absence of green particles in diploids when mating with strains of the same genotype ([*psi*$^-$] [*pin*$^-$]) expressing Sup35(1–61)-GFP and Sup35(1-114)-GFP, respectively (the latter construct could induce [*PSI*$^+$] *de novo* in a small proportion of the diploid cells, which received [*PIN*$^+$] from the [*psi*$^-$] colonies being tested).

Variant typing

The *ade1-14* (UGA) nonsense allele is suppressible by [*PSI*$^+$], which allows the cell to regain the ability for adenine synthesis. This relieves the accumulation of a red pigment, converted from a metabolic intermediate of blocked adenine synthesis. 74-D694 [*PSI*$^+$] colonies are therefore pink or white, and [*psi*$^-$] colonies are red. Some mutant Sup35 proteins may not be compatible with a given [*PSI*$^+$] structure. Their co-expression in the cell weakens the variant, causing the colony to become redder. In severe cases, incompatible mutants may not interact with a prion structure at all. The lack of binding can be observed with GFP fusion constructs.

Ten probes were prepared to differentiate [*PSI*$^+$] variants. The first 5 probes were based on the plasmid YCp33-Hyg-I-SUPF (*URA3*). It contained the hphMX4 marker, followed by a 1.2-Kbp *SUP35* 5'-UTR ("I"), and the Sup35 coding sequence plus 1.1-Kbp 3' UTR. Four Sup35 point mutations, Q15R, S17R, G44R, and G58D, were prepared; their co-expression with endogenous Sup35 in [*PSI*$^+$] cells influenced the color of yeast colonies in a variant-specific

manner. The other 5 probes were based on YEp195-Hyg-Cup1-SUP(1–61)-GFP-T (*URA3*), containing the *hphMX4* marker, a 500-bp *CUP1* promoter, the sequence of the first 61 amino-acid residues of Sup35 fused N-terminally to the green fluorescent protein, and a 240-bp *SUP35* terminator. This set of probes expressed Sup(1–61)-GFP, Sup(1–61)(G20D), (Q23P), (Q23P, N27P), and Sup(1–40)-GFP respectively. The fusion proteins variant-specifically labeled [*PSI*$^+$] particles.

For variant typing, tester cells were prepared by transferring the 10 plasmids to prion-free wild-type or T160M strains. [*PSI*$^+$] colonies were crossed with the testers. The resulting diploids were selected on YPD plates containing G418 and hygromycin B. Variant-specific changes in yeast colony color were discerned directly. GFP labeling was observed by fluorescence microscopy. Cells lacking Ssz1 grow poorly with hygromycin (Kim and Craig 2005). For typing *ssz1*Δ homozygotes, *hphMX4* was replaced with *NatMX4*, and diploid selection was performed on YPD plates containing G418 and clonNAT.

Cytoduction

Cytoduction allows a pair of mating cells to exchange cytoplasm without nuclear fusion. One of the nuclei can then be selectively maintained. Recipient strains were prepared by introducing *kar1Δ15* and *cyh2* mutation. The former impedes nuclear fusion, and the latter confers cycloheximide resistance to allow selection of the nucleus. The strains were cured of all prions as well as mitochondrial DNA ([*pin*$^-$][*psi*$^-$][*rho*^{0}]). Successful mixing of cytoplasm provided recipient strains with healthy mitochondria, allowing them to grow with non-fermentable carbon source. Details for recipient strain construction were reported (Huang et al. 2020).

Recipient cells with a KanMX marker (for example, carrying YCp111-KanMX) were mated with donors on YPD. After 12 h at 30 °C, the cell mix was streaked on YPD plates containing cycloheximide to select colonies with the recipient nucleus. After 2 days at 30 °C, the colonies were replica plated to YPG plates containing glycerol as the carbon source to select cells that have received donor mitochondria (and thus the cytoplasm). The doubly selected colonies were then gridded onto YPD + G418 plates for variant typing. For each cytoduction reaction, at least 2 independent experiments were performed with different donor isolates. Consistent results were obtained and data were combined. For variants whose propagation required episomally expressed Sup35, [*PSI*$^+$] cytoductants were confirmed to be free of the Sup35-encoding plasmid, which could be co-transferred from the donor occasionally.

[*PSI*$^+$] propagation with T160M, *ssz1*Δ, and *upf1*Δ mutants

Class I variants (see below) VK, VL, and B2 in wild-type [*pin*$^-$] donors were cytoduced to mutant recipient cells. Cytoductants were mated with wild-type tester cells expressing Sup(1–61)(G20D)-GFP (for VK, and VL) or Sup(1-114)-GFP (for B2). The diploid colonies were randomly selected, dispersed in water and observed with a fluorescence microscope. More than 1000 cells from each colony were checked for prion particles to estimate the proportion of [*PSI*$^+$] cells.

[*PIN*$^+$] propagation with T160M, *ssz1*Δ, and *upf1*Δ mutants

Wild-type cells propagating Very High and Medium [*PIN*$^+$] were crossed with prion-free triple mutants of the genotype Hsp104^{T160M} *ssz1*Δ:*Nat*MX4 *upf1*Δ:*Kan*MX. Random spores from the resulting diploids were checked on YPD + G418, YPD + clonNAT, and YPD + G418 + clonNAT to identify gene deletions. Colony PCR products were sequenced to identify the Hsp104 allele type. Spores were mated with wild-type testers expressing RNQ1-GFP. Diploid colonies formed were suspended in water. Aliquots were observed with a fluorescence microscope. More than 1000 cells from each colony were checked for prion particles to estimate the proportion of [*PIN*$^+$] cells. The present method was used because transferring [*PIN*$^+$] by cytoduction results in greater residual propagation in T160M and ΔN cells (Huang et al. 2020), which might mask the effect of the deletions.

Western analysis

Cell extracts were prepared with the method of von der Haar (2007). Cells were grown in 10 ml YPAD + G418 at 30 °C till near saturation (OD_{600} ~ 10; cultures stopped growing at OD_{600} ~ 15). Appropriate amounts of the cells (~200 µL) were spun down, resuspended in 100 µL of Lysis Buffer (0.1 N NaOH, 0.05 M EDTA, 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol), and heated at 95 °C for 10 min. Acetic acid (4 M, 2.5 µL) was then added and the mixture was incubated at 95 °C for another 10 min. Samples were then mixed with 5X SDS loading buffer for gel electrophoresis. Proteins were electro-transferred to a PVDF membrane and probed with antibodies listed below. The Amersham Typhoon 5 system was used for image process. Experiments were performed with independent isolates and consistent results were obtained.

Antibodies used: Hsp104: rabbit polyclonal antibody against Hsp104(894–908) (abcam ab69549). Hsp70: mouse polyclonal against chicken Hsp70 (Enzo ADI-SPA-822-F).

Sis1: rabbit polyclonal against yeast Sis1 (custom made). Sup35: rabbit polyclonal against Sup35(55–68) (custom made). Rnq1: rabbit polyclonal against yeast Rnq1 (custom made). Glucose-6-phosphate dehydrogenase (G-6-PDH): used for loading control, rabbit polyclonal against yeast G-6-PDH (Sigma-Aldrich A9521). Secondary antibodies: Alexa Fluor 680 goat anti-rabbit IgG (ThermoFisher A21076) and Alexa Fluor 680 rabbit anti-mouse IgG (ThermoFisher A21065).

Quantitative reverse transcription PCR (RT-qPCR)

Two sets of experiments were performed to compare the relative abundance of $Hsp104^{\Delta N}$ transcripts in cells with and without *UPF1*. The first set used [psi^-][pin^-] strains, and the second used [psi^-] [PIN^+] ([High PIN]) strains. Cells were grown in 10 ml YPAD + G418 until OD_{600} ~ 0.5. Cell pellets were suspended in 400 µl TES buffer (10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% (g/ml) SDS). An equal volume of acid phenol (Sigma-Aldrich P4682) was added. Samples were incubated at 65 ˚C for 60 min, cooled on ice and centrifuged at 1610 x g for 5 min. The aqueous phase was subjected to phenol extraction again, and then chloroform extraction (Sigma-Aldrich 288306). RNA was precipitated with ethanol, washed, and then re-suspended in 50 µl water. cDNA was prepared with Roche Transcriptor First Strand cDNA Synthesis Kit (04379012001) using the anchored-oligo$(dT)_{18}$ primer provided. qPCR was performed with the ThermoFisher QuantStudio real-time PCR system using the SYBR Green dye for dsDNA detection. *ACT1* was used as the internal reference. The difference of cross point difference (ΔΔCq) was used to calculate gene expression ratios. Experiments were performed with 3 biological repeats. Each repeat was done with 3 technical replicates. For *Hsp104*, the qPCR product covered coding sequence 1189–1265. For *ACT1*, 868–940. No nonspecific amplification was detected. RNA alone yielded no PCR products.

Results

Introducing new [*PSI*$^+$] variants

All new [PSI^+] variants reported in the present work are summarized here for easy reference. By transient Sup35 overexpression, 6 new, rare variants were induced *de novo* in T160M cells or in cells containing *ssb1/2*Δ, *ssz1*Δ, and *upf1*Δ mutations (Table 1; Fig. 1). Cells propagating the variants were streaked on agar plates for several times, and single colonies were checked each time to ensure variant-type robustness (see Materials and Methods for the variant-typing protocol). The 6 variants were further transferred to wild-type and T160M cells by cytoduction (i.e. cytoplasm mixing without exchange of nuclei, see Materials and Methods) and re-checked. All of them were able to propagate in at least one of the wild-type or T160M cells without the deletions (Table 2).

[*PSI*$^+$] induction in yeast cells with *ssb1/2*Δ, *ssz1*Δ, or *upf1*Δ mutation

[PSI^+] variants induced in yeast strains with *ssb1/2*Δ, *ssz1*Δ, or *upf1*Δ mutation were selected by nonsense readthrough of the *ade1-14* allele, which encodes a premature UGA stop codon. Two independent experiments were performed for each strain. In first experiments, [PSI^+] colonies were selected by its pink or white color on rich media. (Yeast carrying the *ade1-14* mutation is red on rich media. [PSI^+] suppresses the nonsense mutation and reduces the redness.) In second experiments, [PSI^+] colonies were directly obtained on synthetic media lacking adenine without reference to colony color (cells lacking the prion could not grow). The

Table 1 The host range of the new [PSI^+] variants

Variant	Host of origin	$Hsp104^{T160M}$ propagation	$Hsp104^{WT}$ propagation	$Hsp104^{T160M}$ *ssz1*Δ *upf1*Δ propagation	$Hsp104^{WT}$ *ssz1*Δ *upf1*Δ propagation	Mutability
Ze	$Hsp104^{WT}$*ssb1/2*Δ	unstable	yes	N.D.	N.D.	Not observed
Hdp	$Hsp104^{WT}$*ssz1*Δ	yes	yes	N.D.	N.D.	To VH in WT and T160M
B7	$Hsp104^{T160M}$*ssz1*Δ *upf1*Δ	no	yes	yes	N.D.	To VK in WT To B6 in T160M
V7	$Hsp104^{T160M}$*ssz1*Δ *upf1*Δ	yes	no	yes	no	To VK in WT
V8	$Hsp104^{T160M}$*upf1*Δ	yes	no	yes	no	Hardly observed*
V9	$Hsp104^{T160M}$	yes	no	yes	no	Not observed

N.D.=not determined. WT = wild type cells. T160M = $Hsp104^{T160M}$-expressing cells

* Only one cytoductant was ever observed to propagate [PSI^+] (VK) (in Table 5)

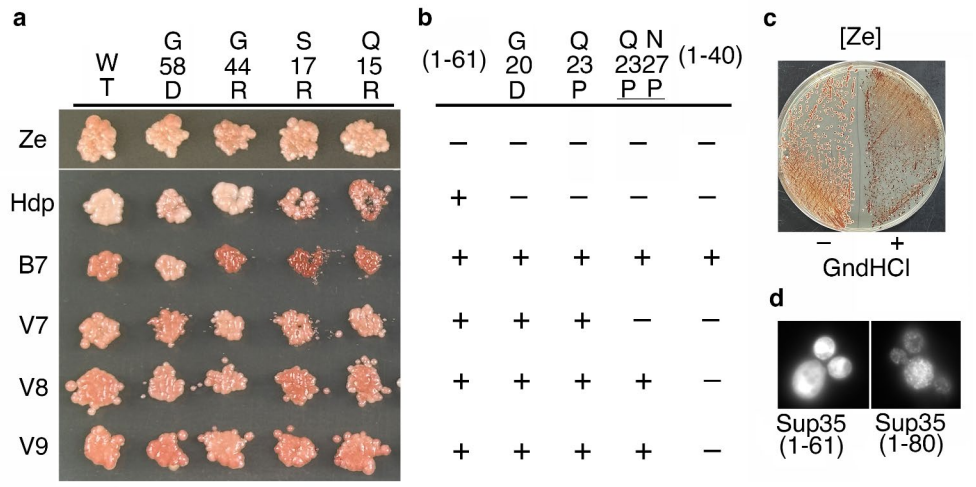

Fig. 1 New [*PSI*$^+$] variants. (**a**) [*PSI*$^+$] variants (labeled on the left) are distinguished by colony colors. Cells are crossed with prion-less testers transformed with plasmids expressing full-length Sup35 or single mutations (top). The diploid colonies show variant-specific color patterns. Ze, Hdp, and B7 are crossed with wild-type testers; V7, V8, and V9 are with T160M testers. (**b**) The variants are distinguished by GFP labeling. Cells are crossed with testers expressing Sup(1–61)-GFP, its single mutants, or Sup(1–40)-GFP (indicated on the top). (+) indicates particulate GFP labeling, as shown in panel (**d**), right; (–) indicates diffused GFP labeling as in (**d**), left. Ze, Hdp, and B7 are crossed with wild-type testers; V7, V8, and V9 are with T160M testers. (**c**) and (**d**) Ze is a proper [*PSI*$^+$] variant although it is unresponsive in variant typing. Ze is cured by 3 mM guanidine hydrochloride (**c**) and 100% labeled by Sup(1–80)-GFP (**d**)

Table 2 Cytoduction of the new variants. The recipient cell type is shown on top

	Hsp104^{WT}	Hsp104^{T160M}
Ze	56/56	20$^{\#}$/58
Hdp	6[Hdp], 18[VH]/24	12[Hdp], 10[VH]/22
B7	24[B7], 2[VK]/28	29[B6]/29
V7	1[VK]/57	24/25
V8	0/30	30/30
V9	0/30	29/29

a/b= [*PSI*$^+$] colony number / total cytoductants variant-typed. The variant type of cytoductants is the same as the donor unless specified. #: tiny [*PSI*$^+$] sectors in otherwise [*psi*$^-$] colonies

majority of [*PSI*$^+$] isolates turned out to be VH, VK, and VL (Table 3). They are well-studied [*PSI*$^+$] variants frequently obtained in wild-type strains. Two new variants did appear. [Ze] and [Hdp] were induced in *ssb1/2*Δ and *ssz1*Δ strains, respectively (Table 3; also see Fig. 1; Table 1). They however could propagate in wild-type cells with normal Ssb1/2 and Ssz1 activities (Table 2). No variant curable by Ssb1/2, Ssz1, or Upf1 was detected.

The above conclusion is supported by an additional observation. [*PSI*$^+$] colonies were mated with wild-type testers carrying a set of reporter plasmids to determine the variant type. All but one colony continued propagating the prion in the diploids where Ssb1/2, Ssz1, and Upf1 functions were restored by complementation. The single exception was a white colony with *SSZ1* deletion, which became red when forming diploids. The colony was transformed directly with plasmids expressing Sup(1–61)-GFP (containing the first 61 amino-acid residues of Sup35 fused with the green fluorescent protein), Sup(1–80)-GFP, and Sup(1-114)-GFP to label possible [*PSI*$^+$] particles in vivo. None of the cells transformed with Sup(1–61)-GFP contained labeled particles. About 30% of the cells transformed with Sup(1–81) or Sup(1-114) had GFP puncta, but the occurrence rate was too low for a [*PSI*$^+$] cell (near 100%). Moreover, the same 30% rate was observed in control experiments where the original deletion strain used for [*PSI*$^+$] induction ([*psi*$^-$] [*PIN*$^+$] Δ*ssz1*) was similarly treated. The puncta were thus considered as an artifact, inadvertently induced by the GFP fusion proteins in a [*PIN*$^+$] background.

To investigate further, the cytoplasm of the white colony was transferred to prion-free recipients that had the same *ssz1*Δ mutation. All resulting cytoductants formed red colonies (30/30). Clearly, the whiteness was not due to [*PSI*$^+$]; it was most likely caused by a recessive mutation on the yeast chromosome.

[*PSI*$^+$] induction in a Hsp104^{T160M} *ssz1*Δ *upf1*Δ strain

[*PSI*$^+$] induction was next performed with T160M cells with *ssz1*Δ and *upf1*Δ mutations. Experiments were executed as described above except that for variant typing, [*PSI*$^+$] colonies selected on adenine-less media were mated with both wild-type and T160M tester cells. The extra work ensured more accurate variant type assignment. Variants that were stable in T160M cells but mutated in wild-type cells (or Hsp104^{WT}/Hsp104^{T160M} heterozygotes) could be confidently identified. The experimental result is listed in Table 3.

Table 3 *De Novo* [*PSI*$^+$] induction

		VH	VK	VL	Ze	Hdp	UnS	B6	B7	V2	V6	V7	total
Hsp104^{WT}*ssb1/2Δ*	#1	70	39	10	1	0	0	0	0	0	0	0	120
	#2	94	19	7	0	0	0	0	0	0	0	0	120
Hsp104^{WT}*ssz1Δ*	#1	63	42	12	0	0	0	0	0	0	0	0	117
	#2	83	28	8	0	1	0	0	0	0	0	0	120
Hsp104^{WT}*upf1Δ*	#1	35	21	4	0	0	0	0	0	0	0	0	60
	#2	52	54	14	0	0	0	0	0	0	0	0	120
Hsp104^{T160M}	#1	37	12	6	0	0	2	1	0	2	0	0	60
ssz1Δ upf1Δ	#2	68	23	21	0	0	0	1	1	2	2	2	120

[*PSI*$^+$] colonies are selected on YPD plates by color in experiments #1 and by growth on synthetic media without adenine in experiments #2

The variants induced here could be grouped into 3 classes, based on compatibilities with wild-type and T160M cells in the absence of the deletions.

Class (I): variants that only propagate with Hsp104^{WT}. Surprisingly, VK and VL variants that normally cannot propagate with Hsp104^{T160M} showed up here (Huang et al. 2020). They were unambiguously typed when colonies were crossed with wild-type testers (i.e. in Hsp104^{WT}/Hsp104^{T160M} heterozygotes) but were cured with T160M testers (Hsp104^{T160M} homozygotes), exactly as expected. In addition, a new variant, B7, was isolated (Fig. 1). It could infrequently change to VK in wild-type cells. When transferred to T160M cells, it mutated to the B6 variant (Huang and King 2020) and disappeared (Tables 1 and 2).

Class (II): variants that only propagate with Hsp104^{T160M}. There were B6, V2, V6 and a new variant, named V7 (see Fig. 1; Tables 1 and 2). They were cured in wild-type cells, but could escape total elimination by changing to VH or VK.

Class (III): variants that can propagate in both wild-type and T160M cells. VH was the most abundant (VH is however less stable in T160M cells, lost in mitosis at a low rate). The UnS variant was also identified, which propagates faithfully with Hsp104^{T160M} but can change to VH and VK in wild-type cells (Huang et al. 2020; King 2022).

In conclusion, double deletion of *SSZ1* and *UPF1* in T160M cells appears to generate an accommodating environment for all [*PSI*$^+$] variants, especially those of Class I, which otherwise can only be maintained with wild-type Hsp104.

The *ssz1Δ upf1Δ* double mutation moderately enhances [*PSI*$^+$] induction in Hsp104^{T160M} cells

The frequency of *de novo* [*PSI*$^+$] induction was estimated in T160M cells with and without the *ssz1Δ upf1Δ* double mutation. The full-length Sup35 protein was overexpressed from a multi-copy plasmid in the cell. Cultures were streak on rich media. About 120 colonies were randomly picked and gridded on agar plates. They were forced to lose the plasmid and then assayed for [*PSI*$^+$] status by colony color. Nearly half of the T160M colonies and 90% of the T160M *ssz1Δ upf1Δ* colonies propagated [*PSI*$^+$] (54/120, 70/119 and 103/120, 107/120, respectively). The frequency of the former was consistent with past observations, and was close to the value obtained in the first paper reporting [*PSI*$^+$] induction by Sup35 (in wild-type strains; Chernoff et al. 1993).

Table 3 shows that 57–62% of the [*PSI*$^+$] colonies induced in T160M *ssz1Δ upf1Δ* cells were VH colonies, and 37−30% were VK or VL colonies. With a 90% overall [*PSI*$^+$] induction rate measured here, there would be about 51–60% of the colonies that propagated VH (57–62% times 90%), 33 to 27% colonies for VK or VL and 10% [*psi*$^-$]

colonies. On the other hand, [*PSI*$^+$] induction in T160M cells generates mostly the VH variant (>90%; Class II variants are also induced, but infrequently; Huang et al. 2020), so there would be roughly 50% VH colonies and 50% [*psi*$^-$] colonies for T160M cells. It seems that the increase in [*PSI*$^+$] induction with the *sszΔ1 upf1*Δ mutation could be largely attributed to the appearance of VK and VL (Class I variants).

[*PSI*$^+$] variants induced in 74-D694 are not cured by Ssz1 or Upf1 overexpression

Son and Wickner (2018, 2020, 2022) used a different setting to induce and select [*PSI*$^+$] variants. The prion was induced by overexpression of Sup35(1-253) with a galactose-inducible Gal1 promoter and selected with the *ura3-14* marker (Monogaran et al. 2006). The inducer and selection marker were conveniently carried by a single centromere-based plasmid p1520 (Wickner et al. 2017). Using this method, [*PSI*$^+$] variants were induced again in the 74-D694 background with *ssz1*Δ, *upf1*Δ, and Hsp104^{T160M} *ssz1*Δ *upf1*Δ mutations. One hundred and twenty Ura$^+$ colonies were randomly selected for each mutant. Figure 2 shows that none of the colonies were cured of [*PSI*$^+$] when mated with wild-type cells overexpressing Ssz1 or Upf1.

The frequencies of [*PSI*$^+$] induction in the 74-D694 strain, reported above, were significantly higher than that observed by Son and Wickner in the BY4742 strain. It can be argued that multiple rounds of induction have occurred in a single 74-D694 cell, and Ssz1-, Upf1-, and Ssb1/2-curable variants were competed out by a co-induced variant, therefore the failure to isolate them. To rule out this possibility, strains carrying p1520 were grown in glucose media, which suppressed the expression of Sup35(1-253), turning off [*PSI*$^+$] induction. Spontaneously occurred Ura$^+$ colonies were then selected. Small colonies were preferentially picked with the

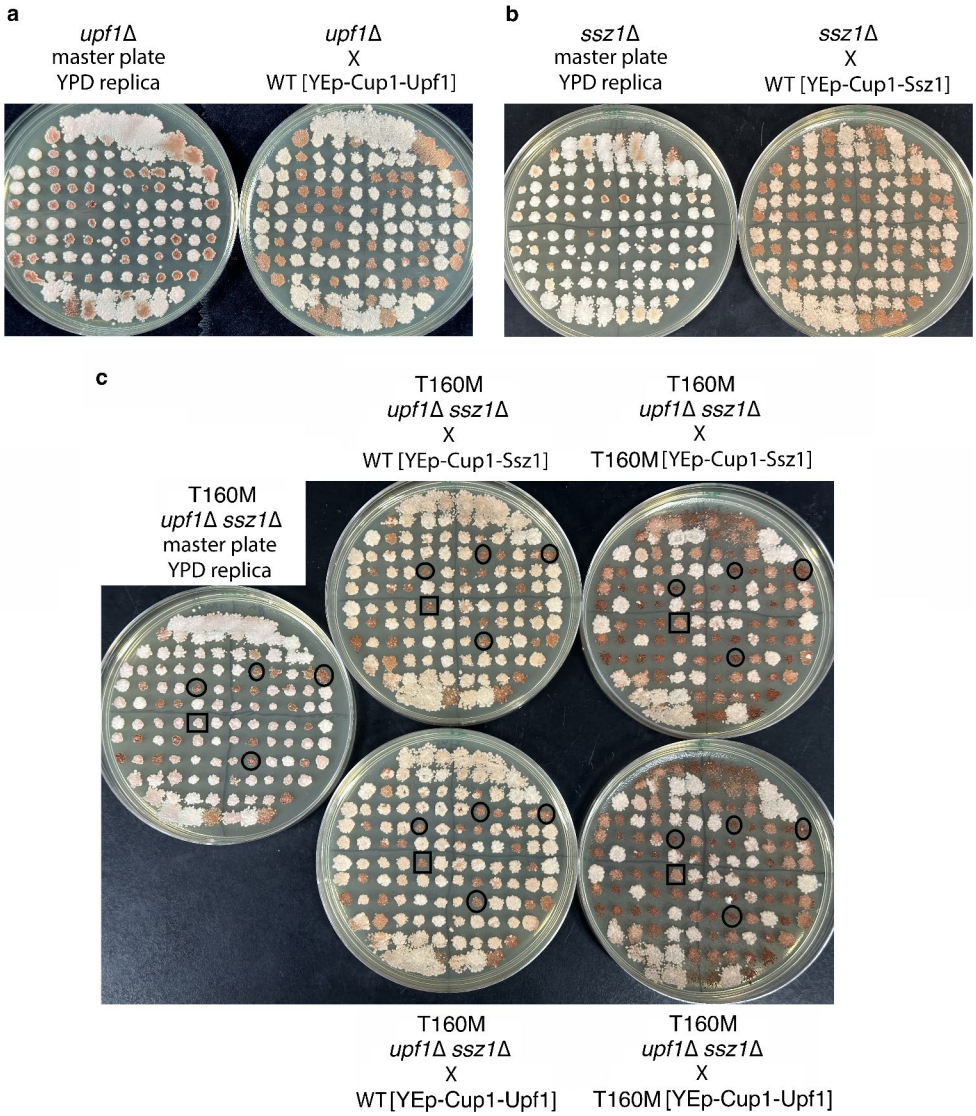

Fig. 2 p1520-induced [*PSI*$^+$] variants are not cured by Ssz1 or Upf1 overexpression in the 74-D694 background. (**a**) [*PSI*$^+$] variants induced in *upf1*Δ cells are not cured by Upf1 overexpression. Randomly picked Ura$^+$ isolates (left) are crossed with wild-type cells overexpress Upf1 (right). No red diploid appears. All isolates on the master plate are variant-typed and thus confirmed to be [*PSI*$^+$] to begin with. (**b**) [*PSI*$^+$] variants induced in *ssz1*Δ cells are not cured by Ssz1 overexpression. Nonsense readthrough is elevated in *ssz1*Δ cells. The elevation is corrected in diploids. (**c**) [*PSI*$^+$] variants induced in Hsp104T^{160M}*upf1*Δ *ssz1*Δ cells are not cured by Ssz1 or Upf1 overexpression. There are 21 Kbar variants (4 random examples are circled), which are cured in T160M homozygotes but not in WT/T160M heterozygotes. Kbar is a Class I variant (see text for definition; King 2022), which can propagate with Hsp104^{WT} but not Hsp104^{T160M} unless *SSZ1* and *UPF1* are deleted. The colony in rectangle propagates B3, which is a Class II variant (i.e. compatible with Hsp104^{T160M} but not Hsp104^{WT} (King 2022). It however can survive in WT/T160M heterozygotes

Table 4 The variant type of spontaneously formed ura+$^{+}$ colonies

		VH	VK	VL	Kbar	[*psi*$^{-}$]	total
Hsp104^{WT}*ssb1/2*Δ	#1	26	2	35	0	27	90
	#2	37	0	20	0	32	89
Hsp104^{WT}*ssz1*Δ	#1	30	3	0	0	53	86
	#2	33	2	1	0	52	88
Hsp104^{WT}*upf1*Δ	#1	9	2	24	0	25	60
	#2	6	12	16	0	26	60
Hsp104^{T160M}	#1	11	1	4	10	151	177
*ssz1*Δ *upf1*Δ	#2	6	5	5	21	142	179

Table 5 Cytoduction of class II variants and VK. The recipient cell type is shown on top

	Hsp104^{T160M}	Hsp104^{WT}	Hsp104^{WT} *ssz1*Δ *upf1*Δ	Hsp104^{WT} *ssz1*Δ *upf1*Δ *ssb1*Δ *ssb2*Δ
V6	53/55	22[VH], 2[VK]/87	5[VH]/29	N.D.
V7	24/25	1[VK]/57	0/29	N.D.
V8	56/56	0/60	0/60	1[VK]/55
V9	45/48	0/60	0/59	0/53
B3	52/57	N.D.*	0/57	0/57
VK	0/49	N.D.	51/56**	51/58

a/b= [*PSI*$^{+}$] colony number / total cytoductants typed. The variant type of cytoductants is the same as the donor unless specified. N.D.= not done. Part of the data for V7 is also shown in Table 2. *See King 2022 for independent data (1[VK]/54). **Colonies contain small red ([*psi*$^{-}$]) sectors

help of a dissection microscope to maximize the isolation of weak [*PSI*$^{+}$] variants (but at the expense of getting false positives, especially given that the background nonsense suppression is elevated in the deletion strains, and there were more than one copy of plasmid-carried *ura3-14* markers in the cell, enhancing the chance of nonsense suppression). No novel variants were uncovered (Table 4). And again, none of the isolated [*PSI*$^{+}$] colonies lost the prion when mated with wild-type cells overexpressing Ssz1 or Upf1 (not shown).

The *ssz1*Δ *upf1*Δ double mutation does not help Hsp104^{WT} to support incompatible variants

I next asked if deleting *SSZ1* and *UPF1* could also enable wild-type cells to propagate Class II variants that are not supported otherwise. Class II Variants V6 and V7 were transferred to recipient cells expressing wild-type Hsp104 and carrying the deletions (Hsp104^{WT} *ssz1*Δ *upf1*Δ). The donor variants were lost in the cytoductants (Table 5). Instead, there were several cell colonies propagating VH, consistent with the fact that V6 can mutate in wild-type cells (King 2022). The result indicated a negative answer.

The appearance of "contaminating" VH colonies in the experiment was not ideal, but besides V6 and V7, other known Class II variants were also mutable. I therefore tried isolating new variants that rarely mutated and only propagated with Hsp104^{T160M}. Variants V8 and V9 were obtained after some efforts (see Fig. 1; Tables 1 and 2). They were duly transferred into the Hsp104^{WT} *ssz1*Δ *upf1*Δ recipient. All of the resulting cytoductants lost [*PSI*$^{+}$] (Table 5). There was an additional consideration. Kiktev et al. (2015) showed that deletion of *SSZ1* causes cytosolic release of ribosome-associated Ssb1/2, and that enhances [*PSI*$^{+}$] curing. To remove this effect, *SSB1* and *SSB2* genes were further deleted from the recipient strain. V8 and V9 still could not propagate (Table 5). As a control, the Class I variant VK was introduced into the quadruple mutant and was observed to stably propagate, as it should (Table 5). Therefore, knocking out *SSZ1* and *UPF1* in a wild-type cell did not make it hospitable for Class II variants.

I next performed cytoduction experiments with B3, which is a Class II variant but can propagate in wild-type cells if the *SUP35* gene is carried by a centromere-based plasmid instead of by the chromosome (i.e. *sup35*Δ [YCp111-Sup35]), indicating that just a tad higher Sup35 expression is able to rescue B3 propagation with Hsp104^{WT} (Huang and King 2020; King 2022). Similar to other Class II variants, B3 could not propagate in the quadruple mutant in the absence of episomal *SUP35* (Table 5). The implication of this finding is discussed below.

Effects of a single gene deletion on [*PSI*$^{+}$] propagation

To gauge individual contributions of *ssz1*Δ and *upf1*Δ for prion propagation, Class I variants VK, VL and B2 (Huang and King 2020) were transferred to Hsp104^{T160M} *ssz1*Δ and Hsp104^{T160M} *upf1*Δ strains by cytoduction. [*PSI*$^{+}$] particles in the cytoductants were detected by labeling with Sup35-GFP fusion proteins (see Materials and Methods for details). For control, the variants were first transferred to T160M recipients without the deletions. As expected, they were unable to propagate (Fig. 3a). Single *upf1*Δ mutation only improved VL propagation. In contrast, *ssz1*Δ allowed most cytoductants to propagate [*PSI*$^{+}$], although some cells in the colonies still lost prion in mitosis, as judged by the absence of labeled particles. The loss was largely mitigated in *ssz1*Δ

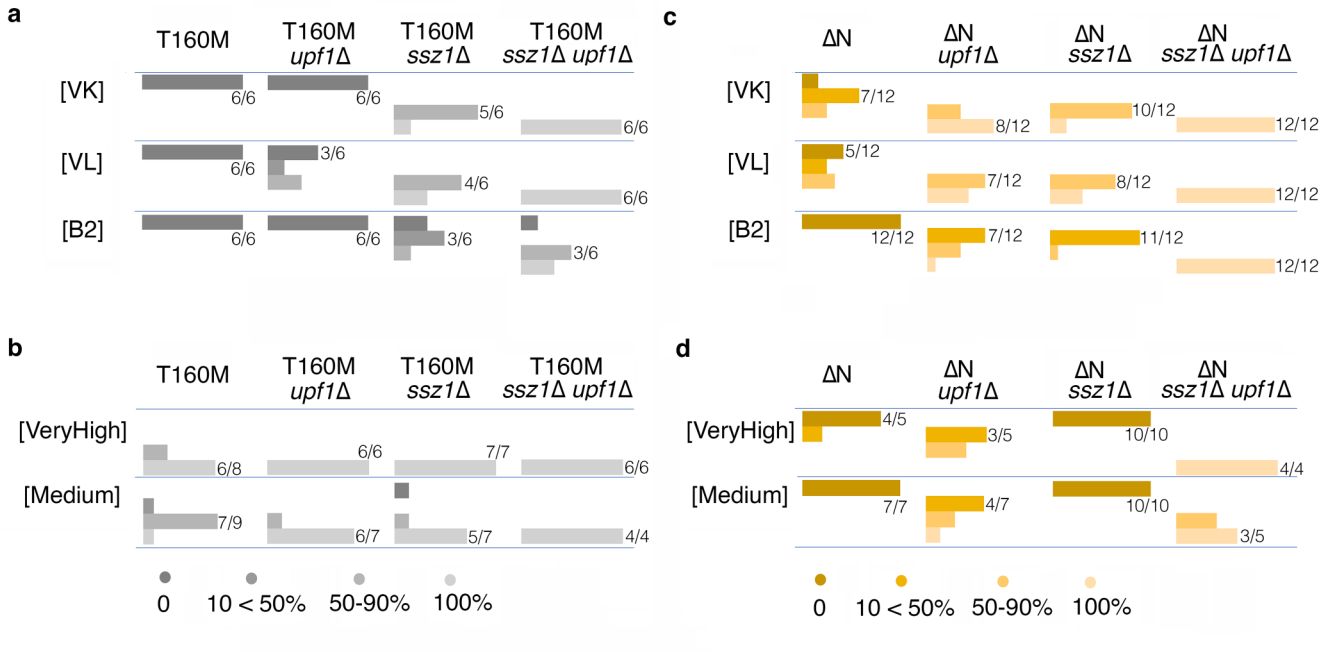

Fig. 3 *ssz1*Δ and *upf*1Δ rescue the propagation of [*PSI*$^+$] and [*PIN*$^+$]. (**a**) [*PSI*$^+$] variants incompatible with Hsp104^{T160M} (indicated on the left) are introduced into hosts with additional *ssz1*Δ and *upf*1Δ mutations (labeled on top). The resulting colonies are checked for [*PSI*$^+$] maintenance. More than 1000 cells of each colony are observed for prion particles by GFP labeling. The proportion of cells containing prion particles are estimated. Proportions are coded by color and by location such that a higher value is represented by a lighter color and a lower position in the entry. For example, regarding B2 propagating in T160M *ssz1*Δ *upf1*Δ (right bottom), among the 6 colonies checked, there is one containing no prion (darkest gray, uppermost position), 3 having 50–90% cells with prion particles (3/6; second lightest, lower middle), and 2 colonies that are 100% labeled (lightest, lowermost). (**b**) [*PIN*$^+$] propagation in T160M cells. (**c**) [*PSI*$^+$] propagation in ΔN cells. (**d**) [*PIN*$^+$] propagation in ΔN cells

*upf1*Δ cytoductants, thus revealing the subtle contribution of *upf1*Δ for all variants (Fig. 3a).

Deleting *SSZ1* and *UPF1* also improves the propagation of [*PIN*$^+$] variants

I next tested if deleting *SSZ1* or *UPF1* could similarly help other yeast prion to propagate with deficient Hsp104. Two [*PIN*$^+$] variants, [Very High] and [Medium], which propagate well in wild-type cells but poorly with Hsp104 mutants, were used for the experiment (Bradley et al. 2002; Huang et al. 2020). Wild-type strains harboring the variants were crossed with T160M *ssz1*Δ *upf1*Δ cells and the resulting diploids were induced to undergo meiosis. Haploid progenies were genotyped and checked for [*PIN*$^+$] by RNQ1-GFP labeling ([*PIN*$^+$] is the prion form of Rnq1). The two [*PIN*$^+$] variants could still propagate in T160M cells, but exhibit some mitotic loss (Fig. 3b). The mitotic instability could be somewhat relieved with additional *ssz1*Δ or *upf1*Δ mutation. For [Very High], either mutation resulted in complete relief. For [Medium], double deletion was needed to achieve total rescue. The results indicate that *ssz1*Δ and *upf1*Δ could also help T160M cells to maintain the [*PIN*$^+$] variants.

The residual propagation of the [*PIN*$^+$] variants in T160M cells can be largely avoided in cells expressing Hsp104$^{\Delta N}$, lacking residues 2- 147 (designated ΔN hereinafter; Huang et al. 2020). The ΔN mutant, like T160M, also exhibits weaker activities, fails to support VK and VL variants, but can nevertheless sustain the propagation of Class II [*PSI*$^+$] variants. Experiments were performed again by crossing the wild-type yeast strains with ΔN *ssz1*Δ *upf1*Δ cells, followed with meiosis and spore analysis.

Deletion of *UPF1* allowed ΔN cells to partially support the [*PIN*$^+$] variants (Fig. 3d). In contrast, none of the ΔN cells with *ssz1*Δ had prion particles. However, *ssz1*Δ might still have some positive influence since the *ssz1*Δ *upf1*Δ double mutation supported [*PIN*$^+$] inheritance much better than *upf1*Δ alone. As an internal control, all haploid cells inheriting wild-type *HSP104* (instead of the Δ*N* allele) propagated [*PIN*$^+$] with 100% GFP-labeling regardless of the deletions. Thus, deleting *UPF1* and *SSZ1* could help [*PIN*$^+$] propagation in ΔN cells.

[*PSI*$^+$] propagation in Hsp104$^{\Delta N}$ backgrounds

To compare [*PSI*$^+$] and [*PIN*$^+$] on a more equal footing, VK, VL and B2 were checked for propagation in ΔN cells, using

cytoduction for prion transfer. Indeed, [*PSI*$^+$] propagation was rescued by *ssz1*Δ and *upf1*Δ mutations. Like the [*PIN*$^+$] variants, VK, VL, and B2 propagated better with *upf1*Δ than with *ssz1*Δ, and the double deletion supported near-perfect propagation (Fig. 3c). The striking similarity between [*PSI*$^+$] and [*PIN*$^+$] reaffirms that general mechanisms are involved in the improvement of prion propagation.

Deletion of *UPF1 and SSZ1* results in higher Hsp104$^{\Delta N}$ expression

Western analysis was performed with cell extracts. The expression level of Hsp104$^{\Delta N}$ correlated nicely with the extent of improvement observed for prion propagation (Figs. 3c and d and 4b). Cells with *SSZ1* deletion showed higher Hsp104$^{\Delta N}$ expression than the control; the expression in *upf1*Δ cells was even higher. And the double deletion resulted in the highest protein level (Fig. 4b). In contrast, Hsp104^{T160M} expression levels were similar across all type of cells. The expressions of Ssa1/2, Sis1, and Sup35 were also analyzed by Western blots; they did not differ significantly among different cell types (not shown).

A careful comparison of Fig. 3a and c reveals that *upf1*Δ had a more pronounced effect in ΔN cells than in T160M cells. This is not unexpected. In constructing ΔN strains (but not T160M strains), a restriction site was introduced between the promoter and the coding sequence of Hsp104$^{\Delta N}$, which inadvertently created an upstream, out-of-frame ATG triplet in the transcript (Fig. 4a). Translation initiated from the fortuitous start would terminate prematurely, making the transcript an NMD target. Knocking out *UPF1* stabilized the transcript. The resulting higher expression of Hsp104$^{\Delta N}$ could compensate for the weak disaggregase activity and help the [*PSI*$^+$] variants to propagate. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) confirmed that *upf1*Δ cells contained about 1.5 times more Hsp104$^{\Delta N}$ transcripts than the Upf1$^+$ counterpart (two sets of experiments were performed, using [*psi*$^-$][*pin*$^-$] and [*psi*$^-$][*PIN*$^+$] strains. The expression ratios were 1.40 ± 0.18 and 1.618 ± 0.31, respectively).

Propagation properties of the D3 variant

Experiments with the D3 variant were informative for understanding the pro-prion effect of the Hsp104^{T160M} *ssz1*Δ *upf1*Δ mutation. There are 4 [*PSI*$^+$] variants, A2, A3, B1, and D3, which can barely propagate in wild-type cells, T160M cells, or cells overexpressing the wild-type Hsp104, but can be maintained in wild-type cells as long as the *SUP35* gene is relocated from the chromosome to a low-copy-number plasmid (Huang and King 2020; King 2022). The 4 variants were cytoduced to Hsp104^{T160M} *ssz1*Δ *upf1*Δ cells; only D3 survived. An interesting situation now arises: in cells with normal Sup35 expression, neither high nor low disaggregase activities afforded by the wild-type Hsp104 and the T160M mutant, respectively, sufficiently support D3. However, the triple mutant propagated the variant. Further experiments showed that D3 was not cured by Ssz1 or Upf1

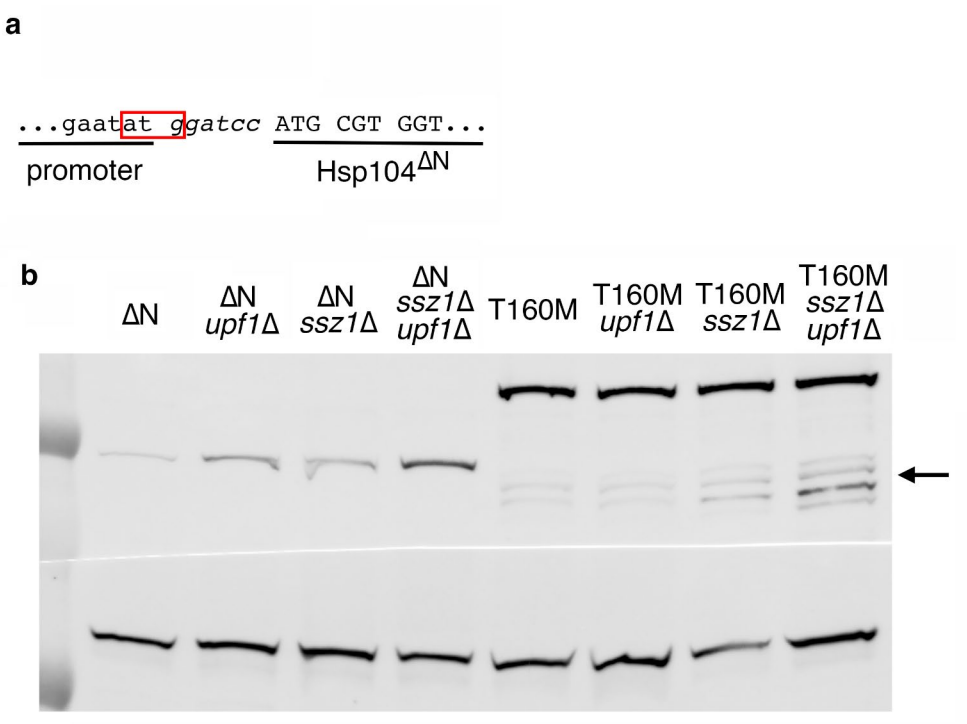

Fig. 4 Hsp104 expression. (**a**) An upstream out-of-frame ATG codon (red rectangle) was fortuitously generated when constructing Hsp104$^{\Delta N}$. The *Bam*HI restriction site is in italics. (**b**) Hsp104 expression in different cell types (indicated on the top) is analyzed by Western blot. ΔN: Hsp104$^{\Delta(2-147)}$. T160M: Hsp104^{T160M}. Loading control: glucose-6-phosphate dehydrogenase (G-6-PDH; on bottom half of the gel). Marker sizes: 100 and 55 KDa. The arrow indicates degradation artefact

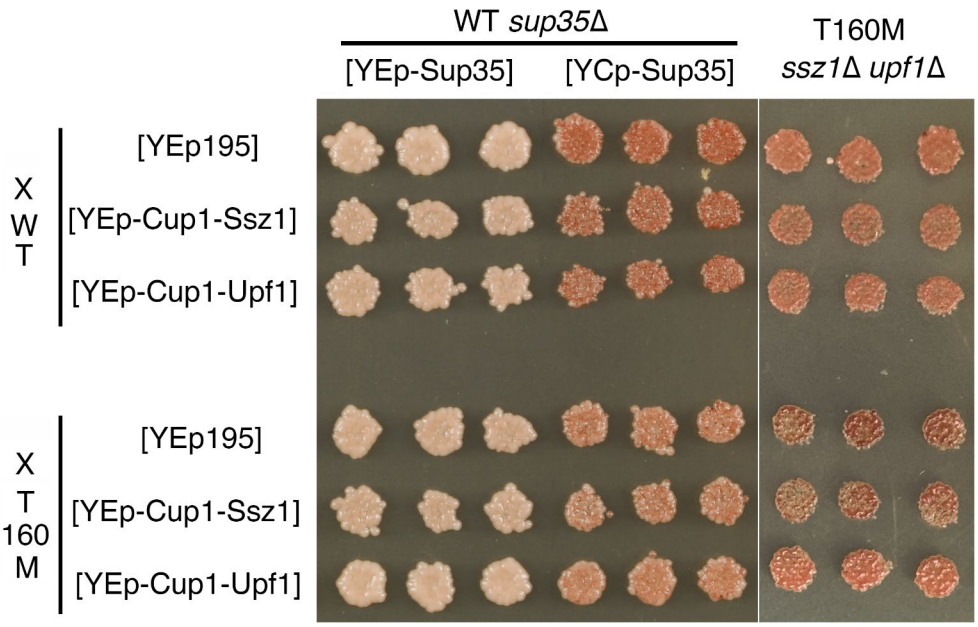

Fig. 5 Ssz1 or Upf1 overexpression does not perturb the D3 variant of [*PSI*$^+$]. D3 can be maintained in both wild-type cells expressing Sup35 from a plasmid and in Hsp104^{T160M}*ssz1*Δ *upf1*Δ mutants with normal Sup35 expression (labeled on the top). The cells are crossed with wild-type or T160M testers overexpresses Ssz1 or Upf1 from a multi-copy plasmid (labeled on the left). No destabilization is observed as colony colors stay the same with or without Ssz1/Upf1 overexpression. Also note that D3 propagates better in Hsp104$^{WT/T160M}$ heterozygotes (lighter color) than in homozygotes. WT: Hsp104^{WT}. T160M: Hsp104^{T160M}. [YEp195]: empty plasmid control

Table 6 Cytoduction of D3

Hsp104^{WT}	Hsp104^{WT} *ssz1*Δ *upf1*Δ *ssb1*Δ *ssb2*Δ	Hsp104^{T160M} *ssz1*Δ *upf1*Δ	Hsp104^{T160M}	Hsp104^{T160M} [YCp-Hsp104^{T160M}]	Hsp104^{T160M} [YCp-Hsp104^{WT}]
3[VH]/59*	0/55	59/60	2/49*	35, 1[VH]/58**	54, 1[VH]/56

D3 is slightly mutable (Huang and King 2020). *: Each of them is a tiny [*PSI*$^+$] sector in a [*psi*$^-$] colony. **: Larger sectors in a [*psi*$^-$] colony

overexpression (Fig. 5), and even without extra Sup35, the variant could be maintained in Hsp104^{T160M} *Ssz1*$^+$ *Upf1*$^+$ cells that co-expressed Hsp104^{WT} (Table 6). Hsp104 is a hexamer. A mixture of hetero-hexamers was generated in the cells. The resulting intermediate activities turned out just right for D3 (see below for more discussion).

Discussion

The starting goal of this work was to isolate and characterize four novel classes of [*PSI*$^+$] variants, reportedly only propagating in yeast backgrounds with *ssb1/2*Δ, *ssz1*Δ, *upf1*Δ and hsp104^{T160M} *ssz1*Δ *upf1*Δ mutations respectively (Son and Wickner 2018, 2020, 2022). I did not find the variants in the 74-D694 strain. A possible explanation for the discrepancy is the genetic background difference, as most of the experiments in the referenced works were performed with the BY4742 background, an S288C derivative. *De novo* [*PSI*$^+$] induction in BY4742 is several orders of magnitude less efficient than in 74-D694 until hsp104^{T160M}, *ssz1*Δ, and *upf1*Δ mutations bring the efficiency up to a comparable level (Son and Wickner 2022). However, it is not clear how different induction efficiencies are related to the distribution of variant types arising. In works by Son and Wickner, significant proportions of [*PSI*$^+$] variants generated in BY4742 backgrounds are the novel variants. In contrast, more than 90% of [*PSI*$^+$] isolates similarly obtained in the 74-D694 strain were VH, VK, and VL, the most studied variants of the wild-type cell. If the mutations merely ease prion detection in BY4742 by elevating baseline nonsense readthrough for [*PSI*$^+$] to ride on, prevailing variant types induced should not differ. Further work is required to clarify and reconcile the different results.

Although not finding the novel variants as reported, I did isolate 6 new variants in the process. All of them occurred infrequently but were able to propagate in cells with normal Ssb1/2, Ssz1, and Upf1 functions. They were named Ze, Hdp, B7, V7, V8, and V9. The first 3 variants propagated in wild-type cells, and V7, V8, and V9 only propagated in T160M cells. The Ze variant is perhaps the most notable among the six. It was curable by guanidine, as [*PSI*$^+$] should, but its propagation was little perturbed by the co-expression of Sup35 N-terminal mutants, as judged by minimal colony color changes in variant typing (Fig. 1). In addition, prion particles of the variant were not labeled in vivo by Sup(1–61)-GFP fusion constructs; for that, Sup(1–80)-GFP was required. The results seemed to suggest that Ze had a more C-terminally located amyloid core, similar to Sup35 amyloids prepared in vitro by Ohhashi et al. (2018). However, recent work showed that Ze particles was labeled by Sup(1–80)(Δ5–10)-GFP (lacking residue 5–10) but not

(Δ5–20), indicating residues between 11 and 20 are required for the structure (data not shown).

We showed previously that the cellular maintenance of [*PSI*$^+$] requires an Hsp104 disaggregase with matching activities (Huang et al. 2020). Many variants (Class I) isolated in wild-type cells are lost in cells expressing Hsp104^{T160M}, which exhibits weaker activities. Conversely, most variants isolated from T160M cells cannot survive in wild-type cells (Class II). It is shown here that T160M cells could support all [*PSI*$^+$] variants if *SSZ1* and *UPF1* were both deleted. A single *ssz1*Δ mutation exhibited partial effect in helping Class I variants to propagate. A single *upf1*Δ mutation showed minimal effect; its influence was largely manifested in the double deletion, which supported [*PSI*$^+$] propagation much better than *ssz1*Δ alone. For Class II variants, *ssz1*Δ *upf1*Δ neither impeded their propagation in T160M cells, nor rescued their curing in wild-type cells. Son and Wickner (2022) reported that deleting *BTN2* and *CUR1* genes from wild-type yeast destabilizes [*PSI*$^+$], and *de novo* [*PSI*$^+$] induction is severely inhibited. In T160M cells, the inhibition can be relieved with an additional *ssz1*Δ mutation or *ssz1*Δ *upf1*Δ double mutation, but not with *upf1*Δ alone. The result bears strikingly similarity to aforementioned observation regarding how *ssz1*Δ, *upf1*Δ, and *ssz1*Δ *upf1*Δ helped the propagation of Class I variants in T160M cells. The relative efficacy of the mutations matches exactly in the two studies.

How do *ssz1*Δ and *upf1*Δ mutations help stabilizing [*PSI*$^+$]? All of the variants induced/studied in the current work were not affected by Ssz1 and Upf1 overexpression, arguing against active curing by the two proteins in 74-D694 (however, this does not necessarily exclude the possibility for other yet uncharacterized variants or for the BY4742 background). The loss of Class I variants in T160 and ΔN cells is due to insufficient fiber fragmentation (Huang et al. 2020). To correct it, more prion particles need to be generated and properly transmitted. In the case of the Hsp104$^{\Delta N}$*ssz1*Δ mutant, there was a slight increase in the expression of Hsp104$^{\Delta N}$ (Fig. 4b), possibly a cellular response to misfolded nascent polypeptides in the absence of ribosome-associated chaperones (*ssz1*Δ causes their dissociation from the ribosome (Kiktev et al. 2015). This caused better fiber fragmentation and hence improved the propagation of the variants (Fig. 3c). For Hsp104$^{\Delta N}$*upf1*Δ, the effect was dominated by the removal of nonsense-mediated degradation of Hsp104$^{\Delta N}$ transcripts, which contains an artefactual out-of-frame ATG triplet right in front of the initiation codon (Figs. 3c and 4). The *ssz1*Δ *upf1*Δ double deletion further improved Hsp104$^{\Delta N}$ expression, enabling near perfect propagation of the variants. For cases involving Hsp104^{T160M}, protein band intensities did not seem to change with *ssz1*Δ and *upf1*Δ mutations except the degradation artefact (Fig. 4b). However, total Hsp104 expression revealed by Western blots may not accurately reflect the elevation in [*PSI*$^+$] fragmentation, which could potentially be achieved through adjustments in the cellular chaperone network in addition to (or instead of) higher Hsp104 expression. For instance, the Hsp104 concentration in T160M cells was higher than that of the Hsp104$^{\Delta N}$ *ssz1*Δ *upf1*Δ triple mutant (Fig. 4b), but it was the latter, not the former, that propagated all [*PSI*$^+$] variants. Other theoretical possibilities, such as the double deletion enhances fiber growth, forces more seeds into daughter cells, or lessens the degradation of amyloid fibers, appear less likely. These mechanisms should also help Class II variants to escape curing by wild-type Hsp104, but such effect was not observed (Table 5).

An idiosyncrasy of the B3 and D3 variant offers an additional argument against the possibility that enhanced fiber growth was a main reason for the pro-prion effect of *ssz1*Δ and *upf1*Δ. It was suggested that deletion of *SSZ1* causes the release of incompletely folded Sup35 from the ribosome, facilitating amyloid formation (Kiktev et al. 2015). Similarly, *UPF1* deletion removes interactions with Sup35 (Son and Wickner 2018), possibly freeing more of the protein for [*PSI*$^+$] growth. B3 and D3 were however not maintained in Hsp104^{WT} *ssz1*Δ *upf1*Δ (*ssb1/2*Δ) cells, although just slightly higher Sup35 expression is known to allow their propagation with wild-type Hsp104 (Tables 5 and 6; Huang and King 2020). This result casts doubt on the significance of the presumed new sources of Sup35 (Tables 5 and 6).

The propagation property of D3 also poses a stringent test for the proposed mechanism that the stabilization of [*PSI*$^+$] in Hsp104^{T160M} *ssz1*Δ *upf1*Δ cells is mainly due to enhanced seed fragmentation. D3 cannot efficiently propagate in wild-type and T160M cells with normal Sup35 expression but could do so in Hsp104^{T160M} *ssz1*Δ *upf1*Δ triple mutants (Table 6; King 2022). It would be problematic for the proposal if D3 was not supported with an enhancement in fiber fragmentation. Reassuringly, as a proof of principle, cells engineered to express both wild-type and the mutant Hsp104 could support D3 (Table 6). The two Hsp104 proteins formed a mixture of hetero-hexamers, which, as a whole, could better fragment D3 but did not dissolve/cure it.

On the surface, D3 behaved like a variant prescribed by Son and Wickner (2022). It was maintained in Hsp104^{T160M} *ssz1*Δ *upf1*Δ cells, mostly cured upon Ssz1 and Upf1 restoration (i.e. in T160M cells), and also eliminated in wild-type cells. Nevertheless, the variant was not affected by excess Ssz1 or Upf1, and contrary to variants reported in the cited work, it was not cured but stabilized in Hsp104$^{WT/T160M}$ heterozygotes (Fig. 5).

Deletion of *SSZ1* and *UPF1* also stabilized some variants of the [*PIN*$^+$] prion. The sub-optimal propagation of Very

High and Medium [*PIN*$^+$] variants in T160M or ΔN cells was remedied with additional deletion of *SSZ1* and *UPF1*. The result indicates the pro-prion effect of *ssz1*Δ and *upf1*Δ is general, not limited to [*PSI*$^+$]. Jay-Garcia et al. (2023) recently demonstrated that deletion of *ZUO1*, a ribosome-associated Hsp40 chaperone that is a partner of *SSZ1*, also facilitates the propagation of some [*URE3*] variants, which cannot propagate in wild-type cells. If *ZUO1* deletion also enhances fiber fragmentation, rather than removes an additional function of curing, the loss of [*URE3*] in wild-type cells should be rescuable with Hsp104 overexpression.

The out-of-frame ATG triplet in Hsp104$^{\Delta N}$ transcript, despite a cloning artefact, was very helpful. The resulting reduction in Hsp104$^{\Delta N}$ expression gave a clean background to assay [*PIN*$^+$] propagation and also enabled useful comparison with results from T160M cells. Out-of-frame ATG codons may be adopted as a general tool for turning down gene expression. Given that the artefactual ATG triplet was a recent realization, it is helpful here to recheck the validity of our previous work which used the same Hsp104$^{\Delta N}$ construct (Huang et al. 2020). There, we showed that the low collective activity of Hsp104$^{\Delta N}$ permits the propagation of Class II variants but impairs the propagation of VK and VL, and excess Hsp104$^{\Delta N}$ can cure them all with good efficiencies. These conclusions remain sound. The new finding however explains why the mutant Hsp104 is less abundantly expressed, and raises the question that how Hsp104$^{\Delta N}$ compares with wild-type and T160M counterparts in terms of intrinsic activities.

In contrast to the observation that *SSZ1* deletion helped the propagation of Class I variants in T160M cells, *ssz1*Δ mutation was reported to disturb the propagation of [*PSI*$^+$] variants in wild-type cells. Kiktev et al. (2015) suggested that deletion of *SSZ1* causes the release of ribosome-associated Ssb1/2 to the cytosol and the freed chaperones compete against Ssa1 for Hsp104 binding, which disturb the propagation of [*PSI*$^+$]. Zhao et al. (2023) recently showed that excess Ssa2 (the 779–6 A allele) can compete against Ssa1 for binding Hsp104 at the N-terminal domain, and the Ssa2-Hsp104 association enhances the trimming and dissolution of prion fibers. Consistently, Chernoff et al. (1999) demonstrated that excess Ssb1/2 helps Hsp104 to cure [*PSI*$^+$]. (Ssa1/2 and Ssb1/2 are all homologous Hsp70 chaperones.) The opposite effect observed here for the Hsp104^{T160M} *ssz1*Δ mutant then should be due to the T160M change, at least for the variants tested by Kiktev et al. The mutation may cause improper interactions with Ssb1/2, or simply removes the N-terminal domain from the action of [*PSI*$^+$] curing (as mentioned earlier, excess Hsp104$^{\Delta N}$, although lacking the N-terminal domain, can still cure VK, VL, and all Class II variants (Huang et al. 2020). In the absence of elevated curing, Class II variants are unperturbed by *SSZ1* deletion and the propagation of Class I variants is positively influenced by the improvement in fiber fragmentation.

In summary, the T160M mutation weakens the disaggregase activity of Hsp104 that otherwise eliminates Class II [*PSI*$^+$] variants. It nevertheless causes insufficient fragmentation of Class I variants, such as VK, VL, and B2, leading to their loss in replicating cells. Additional *ssz1*Δ and *upf1*Δ mutations let Class I variants regain the ability to propagate, making the cell susceptible to all [*PSI*$^+$] variant types. It is perceivable that a similar process could take place in human amyloid diseases. The gradual erosion of cell quality control systems due to aging or somatic mutations could result in booming growth of amyloid species, which in turn exacerbates organ disfunctions.

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Declarations

Competing interests The authors declare no competing interests.

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