Proteolysis suppresses spontaneous prion generation in yeast

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Prions are infectious proteins that cause fatal neurodegenerative disorders including Creutzfeldt–Jakob and bovine spongiform encephalopathy (mad cow) diseases. The yeast [*PSI*$^+$] prion is formed by the translation-termination factor Sup35, is the best-studied prion, and provides a useful model system for studying such diseases. However, despite recent progress in the understanding of prion diseases, the cellular defense mechanism against prions has not been elucidated. Here, we report that proteolytic cleavage of Sup35 suppresses spontaneous *de novo* generation of the [*PSI*$^+$] prion. We found that during yeast growth in glucose media, a maximum of 40% of Sup35 is cleaved at its N-terminal prion domain. This cleavage requires the vacuolar proteases PrA–PrB. Cleavage occurs in a manner dependent on translation but independently of autophagy between the glutamine/asparagine-rich (Q/N-rich) stretch critical for prion formation and the oligopeptide-repeat region required for prion maintenance, resulting in the removal of the Q/N-rich stretch from the Sup35 N terminus. The complete inhibition of Sup35 cleavage, by knocking out either PrA (*pep4*Δ) or PrB (*prb1*Δ), increased the rate of *de novo* formation of [*PSI*$^+$] prion up to ~5-fold, whereas the activation of Sup35 cleavage, by overproducing PrB, inhibited [*PSI*$^+$] formation. On the other hand, activation of the PrB pathway neither cleaved the amyloid conformers of Sup35 in [*PSI*$^+$] strains nor eliminated preexisting [*PSI*$^+$]. These findings point to a mechanism antagonizing prion generation in yeast. Our results underscore the usefulness of the yeast [*PSI*$^+$] prion as a model system to investigate defense mechanisms against prion diseases and other amyloidoses.

The yeast prion [*PSI*$^+$] is a self-propagating amyloidogenic isoform of Sup35 (1–5). Similar to other prions, Sup35 carries a prion-determining domain (PrD),[2] which is required for the formation of self-perpetuating protein aggregates (6–10). PrD consists of a Q/N-rich stretch and adjacent oligopeptide repeat (OR) region. Although both regions are essential for transmission of the [*PSI*$^+$] prion (2, 6), the QN-rich stretch mediates the initial formation of aggregates, whereas the OR region is required for the replication and stable inheritance of these aggregates (11, 12). Modifying these domains may affect the frequency of prion generation. However, relatively little is known about the physiological regulation of the spontaneous *de novo* formation of the [*PSI*$^+$] prion (for a review, see Ref. 2).

Although the process of spontaneous *de novo* prion formation remains elusive, the process of prion induction by Sup35 overproduction is associated with the actin cytoskeletal networks and components of the endocytic/vacuolar pathway (13, 14). Overexpression of Sup35 or its PrD gives rise to peripheral ring-like aggregates along the cell membrane. The peripheral ring then collapses and is transported to surround the vacuole (15). The cells with the ring-like aggregates generate the [*PSI*$^+$] prion. Assembly of the endocytic vesicles and their movement toward the endosomal/vacuolar system involves actin polymerization/depolymerization. The cytoskeleton-assisted generation of aggregates of prion-forming proteins resembles the formation of aggresomes, which accumulate misfolded proteins in mammalian cells.

Here, we show that proteolysis suppresses the spontaneous *de novo* formation of the [*PSI*$^+$] prion. The PrA–PrB proteases mediate the cleavage of Sup35 between the Q/N-rich stretch and oligopeptide-repeat region, resulting in the removal of the Q/N-rich stretch, critical for prion formation, from the N terminus of Sup35. This leads to inhibition of the spontaneous *de novo* formation of [*PSI*$^+$]. However, the amyloid conformers of Sup35 in [*PSI*$^+$] strains were not cleaved and preexisting [*PSI*$^+$] was not eliminated by the activation of the proteases. Our results indicate that proteolysis acts as a defense mechanism against prion generation in yeast.

Results

A short isoform of Sup35 appears during fermentative growth in glucose

In the course of investigating the molecular dynamics of Sup35, we noted that a short isoform of Sup35 appears during

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This article contains supplemental Figs. S1–S4 and Tables S1–S4.

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[2] The abbreviations used are: PrD, prion-determining domain; OR, oligopeptide repeat; PrA, proteinase A; PrB, proteinase B; PrC, proteinase C; CHX, cycloheximide; Sup35s, short isoform of Sup35; APP, amyloid precursor protein; Ub, ubiquitin; V-ATPase, vacuolar ATPase.

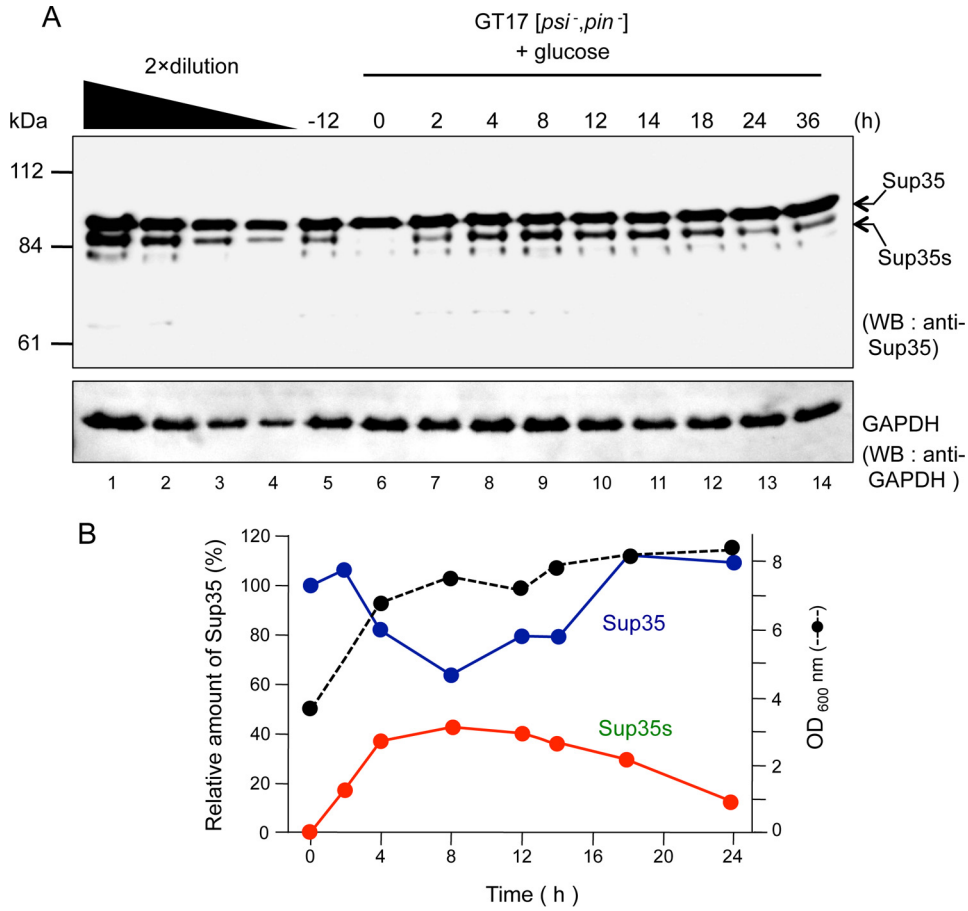


Figure 1. A short isoform of Sup35 appears during fermentative growth in glucose. *A,* GT17 [*psi*$^-$ *pin*$^-$] cells grown in the mid log-phase (*lane 5*) were starved in YPA medium for 12 h (*lane 6*), and cultured in YPDA. Cells were harvested at the specified time points (*lanes 7–14*) and analyzed by Western blotting (*WB*) using anti-Sup35 and anti-GAPDH. The *four leftmost lanes* indicate 2-fold dilutions of the protein to confirm that the condition used was semi-quantitative. *B,* the protein levels of Sup35 (*blue*) and Sup35s (*red*) were measured by MultiGauge and normalized to that of GAPDH, and the normalized Sup35 level (0 h) was defined as 100%. *Dotted line* indicates the A_{600} of the yeast culture.

growth in nutrient-rich media. When cells were cultured with 2% glucose after 12 h of starvation, the short isoform of Sup35 (hereafter denoted as Sup35s), with an apparent molecular mass of ~5 kDa less than that of full-length Sup35, appeared within 2 h after the addition of glucose (mid or late log-phase), peaked at 8 h (stationary phase), and then returned to basal levels according to the depletion of glucose over 24 h of culture (Fig. 1, *A* and *B*). The transiently increased appearance of this isoform was inversely correlated with a transient decrease in the amount of full-length Sup35.

Sup35 is cleaved through the activation of PrA–PrB proteases

We surmised that the observed appearance of Sup35s was due to proteolysis. Thus, we examined the effects of various protease inhibitors including the serine protease inhibitor PMSF, cysteine protease inhibitor E-64, and proteasome inhibitor MG132. Among these inhibitors, PMSF completely inhibited the glucose-induced appearance of Sup35s (Fig. 2*A*). These results suggest that Sup35 is proteolytically cleaved during growth in glucose media, and a serine protease is involved in this process. Therefore, we screened a collection of budding yeast knockout strains (16) for the mutants of serine protease responsible for the cleavage of Sup35. As shown in Fig. 2*B*, Sup35s was still observed in strains *nma111*Δ, *imp2*Δ, *ste13*Δ, and *pcp1*Δ, whereas we could not detect any Sup35s in *prb1*Δ (Fig. 2*B*). Proteinase B (PrB), the protein product of the *PRB1* gene, has been identified as a subtilisin/furin class serine protease that matures in the vacuoles (17). Proteinase A (PrA), encoded by *PEP4*, catalyzes the cleavage of PrB zymogen (Pro-PrB) to produce the active mature form of PrB, whereas PrB catalyzes the cleavage of proteinase C (PrC) zymogen (Pro–PrC), which is encoded by *PRC1*, to produce active PrC (18). Notably, *pep4*Δ, but not *prc1*Δ, abolished Sup35s, leading to the same phenotype as *prb1*Δ (Fig. 2*C*). These results indicate that PrA–PrB proteases mediate Sup35s production.

Cleavage of Sup35 occurs in a manner dependent on translation and independently of autophagy

The involvement of vacuolar proteases PrA–PrB in Sup35s production implies that Sup35 is delivered to the vacuole by a process of autophagy and cleaved in the vacuole. To examine these possibilities, we tested if Sup35s production occurs in autophagy-deficient strains, in which the core autophagy factors *ATG3* and *ATG4* are knocked out. As shown in Fig. 3*A*, cleavage of Sup35 was observed equally in both wild-type and autophagy-deficient strains. These results indicate that the Sup35 cleavage is not the result of autophagy and the auto-

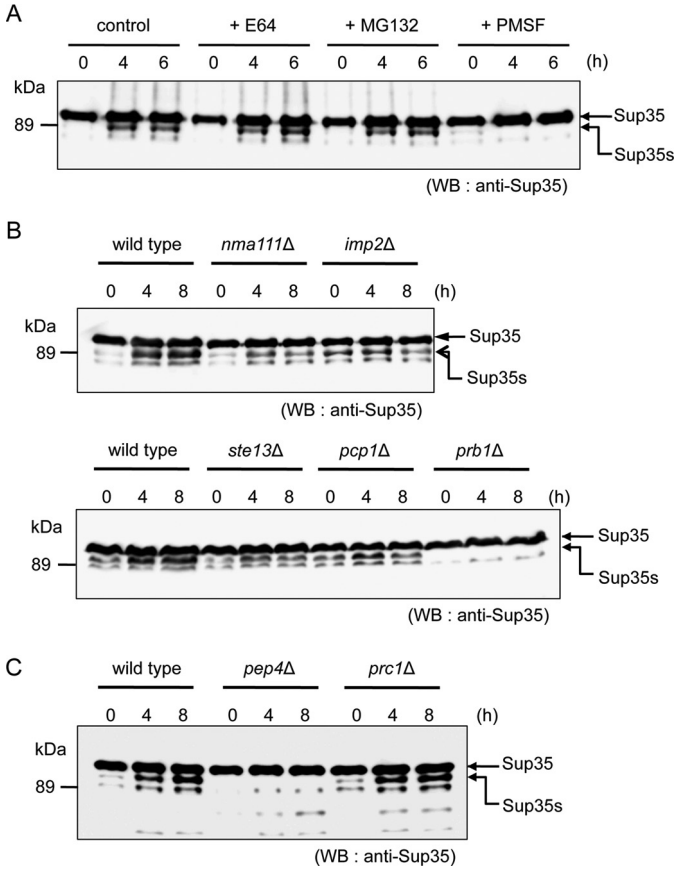


Figure 2. Sup35 is cleaved through the activation of PrA–PrB proteases. *A,* glucose-starved GT17 [*psi*$^{-}$ *pin*$^{-}$] cells were cultured in YPDA containing either 0.5 mM PMSF, 20 μM E64, 100 μM MG132, or their vehicle control. Cells were harvested at the specified time points and analyzed by Western blotting (*WB*) using anti-Sup35. *B,* wild-type (BY4741) and five serine protease knock-out strains (*nma111*Δ, *imp2*Δ, *ste13*Δ, *pcp1*Δ, and *prb1*Δ) were treated as in *A*. *C,* Prb1-related protease knock-out strains (*pep4*Δ, *prc1*Δ) were analyzed as in *B*.

phagic process is not primarily responsible for the cleavage of Sup35. Next, we examined if cleavage occurs in the vacuole. A recent study showed that C terminally tagged PrB purified from the *pep4*Δ strain is active and can cleave the histone H3 *in vitro* (19). Thus, PrB protein was purified from the lysate of the *pep4*Δ strain carrying a single copy plasmid expressing PRB1-FLAG. The anti-FLAG affinity-purified fraction contained a major 45-kDa protein recognized by both anti-FLAG and anti-PrB antibodies (supplemental Fig. S1). Unexpectedly, the proteolytic activity of the purified PrB, which was assessed using the azocoll assay, showed that the optimum pH for the cleavage reaction was around pH 6.5 and the activity of the purified PrB decreased dramatically at an acidic pH below 5.5 (supplemental Fig. S2). Under the optimum conditions, we incubated yeast lysate from the *prb1*Δ strain with or without purified PrB. The purified PrB could recapitulate Sup35 cleavage *in vitro* to produce a small isoform with a molecular weight corresponding to Sup35s observed *in vivo* (Fig. 3*B* and supplemental Fig. S3). The cleavage activity of the purified PrB was inhibited by PMSF (Fig. 3*C*). In sharp contrast, the purified PrB could hardly cleave Sup35 at a vacuolar acidic pH (pH 5.0) (Fig. 3*D*). These results suggest that Sup35 cleavage by PrB occurs outside the vacuole.

Because Sup35 functions as a translation termination factor, we next examined if cleavage occurs at or near the translation site. To test this, we first examined the effect of the protein synthesis inhibitor cycloheximide (CHX) on the cleavage of Sup35. As shown in Fig. 3*E*, CHX completely inhibited the cleavage of Sup35. The result suggests that Sup35 cleavage occurs during or coupled with translation. To address this possibility, we next examined the localization of Sup35s in ribosomal fractions by polysome profile analysis. Fig. 3*F* shows that in wild-type cells, Sup35s along with full-length Sup35 localized in the polysomal fractions. The band corresponding to Sup35s was confirmed from the comparison with prb1-deficient cells (Fig. 3*F*, *right panel*). The result indicates that Sup35s is produced during translation or before Sup35 is transported to the polysome. A previous study indicated that misfolded Sup35 aggregates generated in the process of prion induction by Sup35 overproduction are transported to the vicinity of the vacuole through the actin cytoskeletal networks and the endosomal/vacuolar pathway (13). Therefore, we examined the effect of the disruption of *SLA1* or *END3* on the cleavage of Sup35. As shown in Fig. 3*G*, Sup35 cleavage was still observed in the mutants defective in the actin cytoskeletal networks and endosomal/vasuolar pathway. Taken together, these results are consistent with the idea that Sup35 cleavage occurs outside the vacuole.

Sup35 is cleaved at the N-terminal prion domain

To determine whether Sup35 is cleaved at the N terminus or C terminus, we engineered Sup35 derivatives with the N-terminal or C-terminal FLAG tags. The engineered Sup35 protein was expressed in cells and detected by Western blotting using an anti-FLAG antibody (Fig. 4*A*). The band corresponding to Sup35s was detected for C terminally tagged Sup35 at 6 h after the addition of glucose (late log-phase cells), indicating that the glucose-induced cleavage of Sup35 occurred at the N terminus. We thus purified Sup35s and performed N-terminal Edman sequencing to identify the cleavage site in Sup35. N-terminal sequencing by the Edman degradation method gave the sequence for the first 5 amino acids as AQPAG, which corresponded to the 39th-43rd amino acids of Sup35 (Fig. 4*B*). The identified cleavage site (Ala^{39}) was located exactly between the Q/N-rich stretch and OR region of Sup35. As the Q/N-rich stretch is essential for the induction of [*PSI*$^{+}$] (7, 11) by overproduced Sup35, these data suggest that removal of the Q/N-rich stretch by the glucose-induced cleavage of Sup35 may decrease the *de novo* formation of [*PSI*$^{+}$].

The PrA-PrB-mediated Sup35 cleavage negatively regulates de novo formation of [PSI^{+}]

To test the above prediction, we first examined the frequencies of spontaneous [*PSI*$^{+}$] formation in the early and late log-phase cells of strains containing another prion, [*PIN*$^{+}$], which promotes the *de novo* formation of [*PSI*$^{+}$] (20). In the late log-phase cells, active mature PrB was produced and Sup35 cleavage occurred, whereas in the early log-phase cells, both the active mature PrB and Sup35s were hardly observed (Fig. 5*A*). As expected, the frequency of [*PSI*$^{+}$] appearance was signifi-

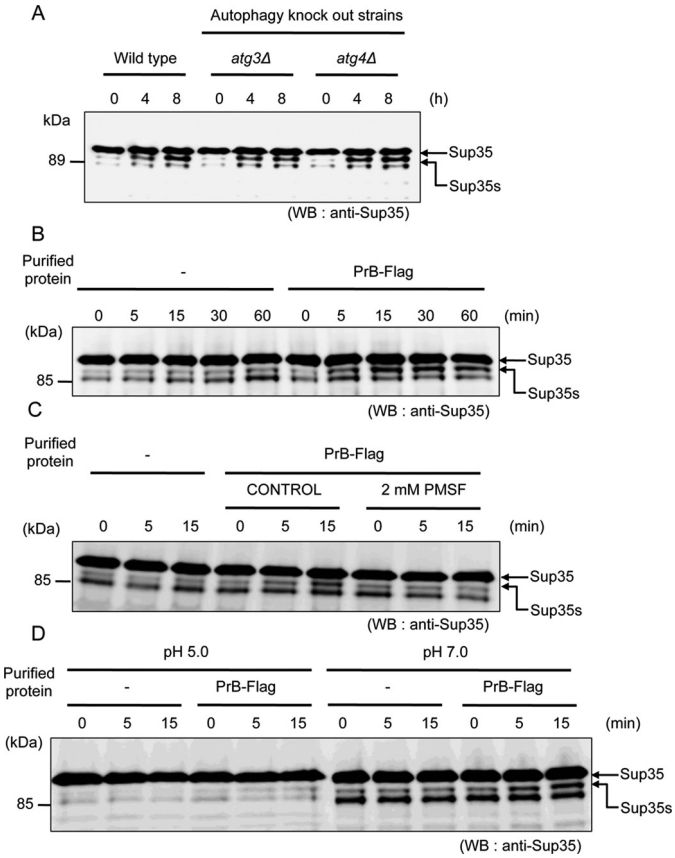


Figure 3. Sup35 cleavage occurs in a manner dependent on translation and independently of autophagy. *A,* wild-type (BY4741) and two autophagy-deficient mutant strains (*atg3*Δ and *atg4*Δ) were starved in YPA for 12 h and cultured in YPDA. Cells were harvested at the specified time points and analyzed by Western blotting (*WB*) using anti-Sup35. *B,* PrB was purified from the culture supernatant of a *pep4*Δ strain carrying a PRB1-FLAG single copy plasmid. *In vitro* Sup35 cleavage reactions were performed by incubating anti-FLAG affinity purified PrB with yeast cell lysate from *prb1*Δ knock-out strain at 30 °C. An aliquot of the reaction mixture was collected at the indicated time points and analyzed by Western blotting using anti-Sup35. *C, in vitro* Sup35 cleavage reactions were performed as in *B* except treatment was with 2 mM PMSF or vehicle control. *D, in vitro* Sup35 cleavage reactions were performed as in *B,* except that the condition was at acidic pH (pH 5.0) or neutral pH (pH 7.0). *E,* glucose-starved GT17 [psi^- pin^-] cells were cultured in YPDA containing either 100 μg/ml of CHX or its vehicle control. Cells were harvested at the specified time points and analyzed by Western blotting using anti-Sup35. *F,* cell extracts prepared from wild-type (OT60) and *prb1*Δ (yAO66) were subjected to the polysome profile analysis. *Top*, absorbance at 254 nm. *Bottom*, Western blot analysis using anti-Sup35. *G,* wild-type (BY4741) and two deletion strains of actin-cytoskeleton and endosomal/vacuolar pathways (*sla1*Δ and *end3*Δ) were treated as in *A*.

cantly higher in the early log-phase cells as compared with the late log-phase cells (Fig. 5*B*). Next, we examined the frequencies of spontaneous [PSI^+] formation in the *pep4*Δ and *prb1*Δ strains, defective in the cleavage of Sup35 (see above, Fig. 2, *B* and *C*). In both strains, active mature PrB was not detected (Fig. 5, *C* and *E*, *right panel*). In the *pep4*Δ cells, inactive pro-PrB was produced, but could not be processed to the active mature PrB (Fig. 5*C*, *right panel*). Consistent with this observation, Sup35s was not detected, even in the late log-phase cells (Fig. 5, *C* and *E*, *left panel*). As expected, the frequency of [PSI^+] appearance was significantly higher in each of the *pep4*Δ and *prb1*Δ strains, as compared with the wild-type strain (Fig. 5, *D* and *F*). However, in *prc*Δ cells, which are deficient in another vacuolar protease PrC but normally express mature PrB, Sup35s was detected (Fig. 5*G*) and the frequency of [PSI^+] appearance was equivalent to that in the wild-type strain (Fig. 5*H*). In contrast, the strain overexpressing *PRB1* constantly produced active mature PrB to generate Sup35s even in the early log-phase (Fig. 5*I*) and exhibited a significantly reduced frequency of spontaneous [PSI^+] formation (Fig. 5*J*). Taken together, these results indicate that the PrA–PrB-mediated proteolysis of Sup35 suppresses the *de novo* generation of the [PSI^+] prion.

Sup35s itself does not antagonize prion generation

Next, we examined if production of the cleaved Sup35 at Ala^{39} (39ASup35) exerts an effect on the frequencies of spontaneous [PSI^+] formation. We constructed plasmids expressing ubiquitin (Ub)-tagged wild-type Sup35 (1MSup35) or amino-terminal 38 amino acid-deleted Sup35 (39ASup35). As described previously, when the eRF3 constructs are expressed in cells, the Ub moiety is removed by multiple ATP-dependent protease, and a Sup35 fragment without Ub can be produced in yeast cells (Fig. 6*A*). As shown in Fig. 6*B*, the expression of 1MSup35 doubled the amount of Sup35 in cells and increased the frequencies of spontaneous [PSI^+] formation, as expected from a previous report (7, 21). However, the expression of 39ASup35 had no significant effect on spontaneous [PSI^+] formation compared with the control case with vector alone (Fig. 6*C*). These results indicate that the production of Sup35s itself does not antagonize prion generation.

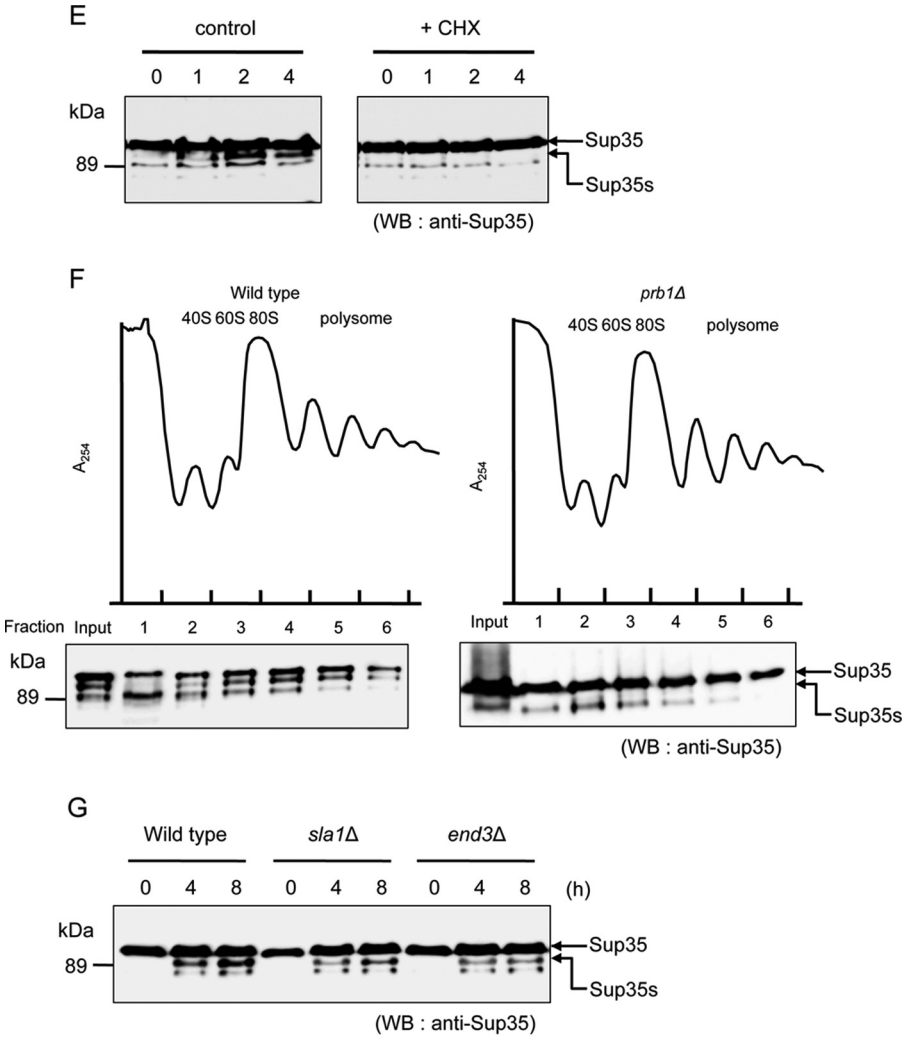


Figure 3—*continued*

PrB activation neither cleaves the amyloid conformers of Sup35 nor eliminates preexisting [PSI$^+$]

Finally, we determined whether the activation of proteases could cure preexisting [*PSI*$^+$]. As Sup35 protein may form distinct variants of the [*PSI*$^+$] prion that differ from each other in suppressor efficiency and mitotic stability (2, 7), we checked two [*PSI*$^+$] variants, designated here as "weak" and "strong." At normal levels of PrB, we did not detect any cleavage of Sup35 in the [*PSI*$^+$] cells under conditions that induced Sup35s in the [*psi*$^-$] cells (Fig. 7*A*). When *PRB1* was overexpressed, still no cleavage was detected in the extracts of the strong [*PSI*$^+$] strain, and also some minor cleavage (less than that in the [*psi*$^-$] background) was detected in the extracts of the weak [*PSI*$^+$] strain (Fig. 7*B*). This was despite the fact that mature PrB was produced at levels comparable with the isogenic [*psi*$^-$] overexpressor (Fig. 7*C*). These data are consistent with the previous observations showing that most Sup35 is aggregated in cells containing weak [*PSI*$^+$], and almost all Sup35 is aggregated in cells containing strong [*PSI*$^+$] (7, 22), and that the prion form of Sup35 is more protease-resistant than the non-prion form (23, 24). Notably, PrB overexpression had no effect on [*PSI*$^+$] maintenance as it did not cause the appearance of red ([*psi*$^-$]) colonies (Fig. 7*D*). From these results, we conclude that the activation of PrA–PrB proteases suppresses the *de novo* generation of [*PSI*$^+$], but cannot cure preexisting [*PSI*$^+$].

Discussion

In this study, we have shown that Sup35 is cleaved at its amino-terminal prion domain during growth in glucose media and the cleavage is mediated by the PrA–PrB proteases. An *in vitro* cleavage experiment showed that PrB plays an essential role in this reaction. The identified cleavage site Ala^{39} is located exactly between the Q/N-rich stretch and oligopeptide-repeat region of Sup35. Therefore, the cleavage releases the Q/N-rich stretch, which is essential for the *de novo* [*PSI*$^+$] formation (10). These data imply that the PrA–PrB-mediated cleavage of Sup35 may affect the *de novo* [*PSI*$^+$] formation. Indeed, we have shown that PrA–PrB negatively regulates the *de novo* generation of [*PSI*$^+$]. Although we cannot totally rule out the possibility that the PrA–PrB also targets proteins other than Sup35 to suppress [*PSI*$^+$] formation, it is reasonable to think that PrA–PrB regulates [*PSI*$^+$] formation at least in part through the cleavage of Sup35. It is also possible to assume that production of the cleaved Sup35 (Sup35s) itself exerts an effect on the for-

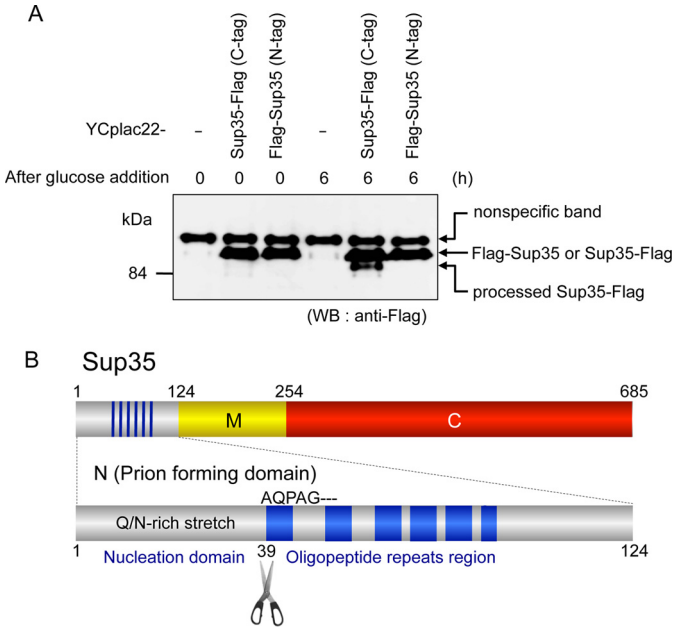


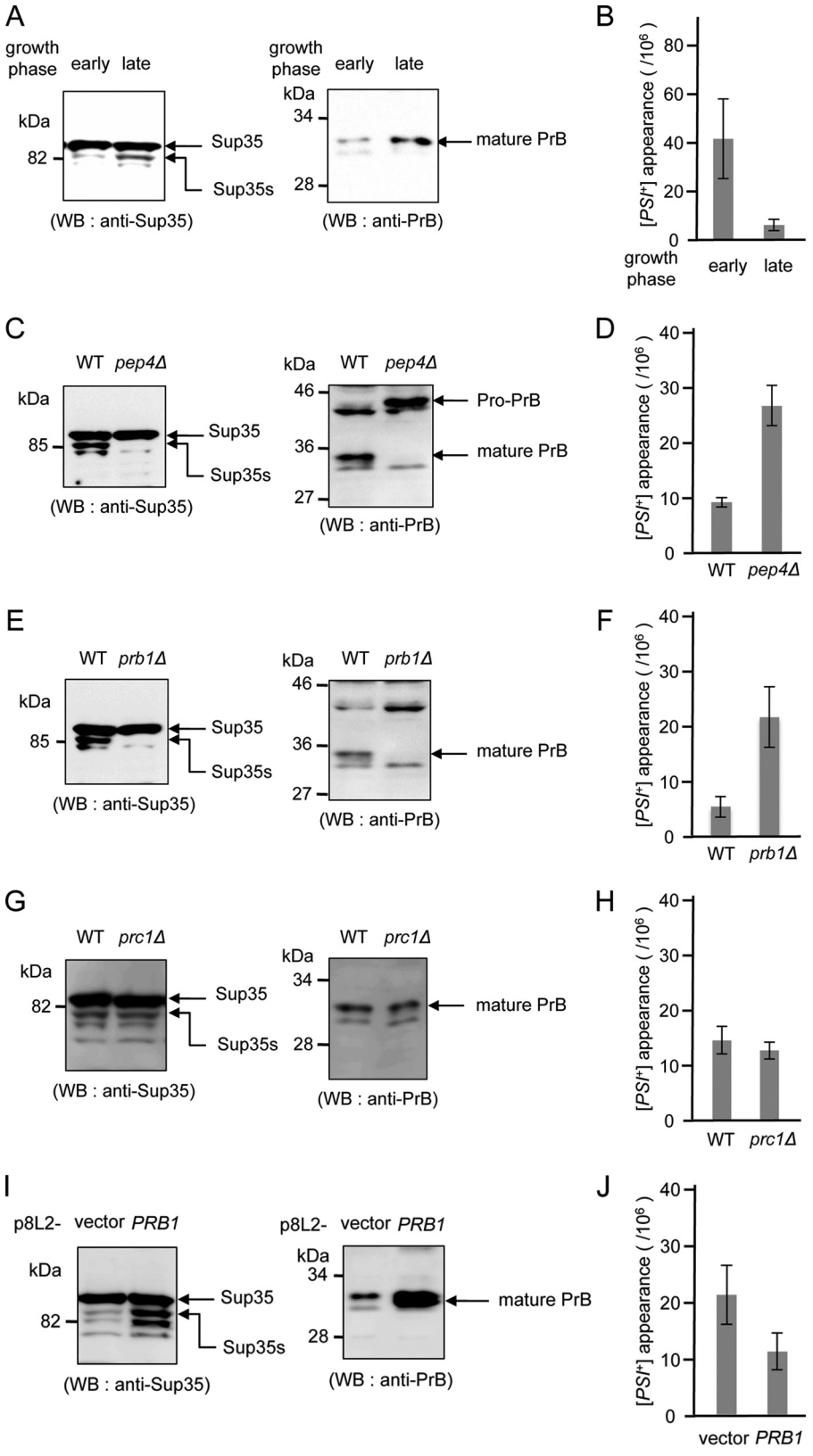
Figure 4. Sup35 is cleaved at the N-terminal prion domain. *A,* YCplac22-SUP35-Flag(C-tag) (yAO149) and YCplac22-Flag-SUP35(N-tag) (yAO150) transformants were cultured in YPDA after a 12-h starvation in YPA. Cells were harvested at the specified time points and analyzed by Western blotting (*WB*) using anti-FLAG. *B,* schematic representation of Sup35. The N-terminal prion domain (1–124) consists of a Q/N-rich stretch and oligopeptide repeats region. Cleavage occurs at Ala[39].

mation of [*PSI*$^+$]. However, the expression of 39ASup35 had no significant effect on the spontaneous formation of [*PSI*$^+$], which was measured by the adenine assay. Previous study demonstrated that 39ASup35 is aggregation-defective (11). We have also observed that 39ASup35 is functionally compromised when expressed in sup35Δ cells (data not shown). Therefore, it is unlikely that production of the cleaved Sup35 itself affects formation and phenotype of [*PSI*$^+$]. On the other hand, we also showed that activation of the PrB pathway neither cleaves the amyloid conformers of Sup35 nor eliminates pre-existing [*PSI*$^+$]. These results indicate that the protease-mediated anti-prion system identified in this study prevents prion generation but cannot cure preexisting prions.

It remains unclear which cellular compartment houses the PrA–PrB-mediated cleavage of Sup35. In this study, we provide evidence showing that: (i) the cleavage is dependent on translation, and (ii) the cleaved product Sup35s localizes in the polysome. These results strongly suggest that the translation termination factor Sup35 is cleaved on actively translating ribosomes. Although PrB is generally thought to be involved in vacuolar proteolysis, a recent study reported that PrB cleaves histone H3 in the nucleus (19). This indicates that PrB also functions outside the vacuole. In the yeast vacuolar lumen, the pH is maintained as acidic (pH 5.0) (25) and the pH optimum of the major vacuolar proteases (*e.g.* PrA, etc.,) is acidic, corresponding to the vacuolar pH (26). However, it was surprising that the pH optimum of the proteolytic reaction measured using the purified PrB is about neutral, which was also mentioned previously by Jones' group (27). Consistent with this, we have shown that Sup35 could hardly be cleaved under acidic conditions *in vitro*. These results further support the idea that the cleavage of Sup35 occurs outside the vacuole. Although the process of spontaneous *de novo* prion formation remains elusive, Sup35 aggregates generated in the process of prion induction by Sup35 overproduction are associated with the actin cytoskeletal networks and components of the endosomal/vacuolar pathway (13, 14). In this study, we have shown that the cleavage of Sup35 could still be observed in deletion mutants lacking the proteins Sla1 or End3, which are involved in the actin cytoskeletal network and endosomal/vacuolar pathway. Therefore, it is reasonable to assume that misfolded Sup35, produced either after overproduction or accidentally during physiological processes (*i.e.* translation termination and mRNA deadenylation), is proteolytically cleaved before being transported via the actin cytoskeletal network and endosomal/vacuolar pathway toward vacuoles.

It also remains to be determined why the cleavage of Sup35 is linked to the growth in glucose media. The expression of PrB is known to be regulated by glucose. *PRB1* transcription is barely detectable during the early log-phase and de-repressed when the cells have exhausted the glucose supply and enter the stationary phase (28, 29). The level of PrB enzymatic activity measured under optimal (neutral) conditions increases as the cells progress from the log-phase to the stationary-phase. However, this might not reflect all of the PrB activity in cells. A recent study reported that vacuolar ATPase (V-ATPase) regulates the cytosolic pH in response to the availability of a carbon source (30). The cytosolic pH is around neutral (pH 6.7∼7.0) during the exponential growth phase and mid to late exponential phase (that is, before glucose is depleted) and then is decreased to the acidic level in the stationary-phase (31). Thus, the cytosolic PrB is thought to be active in log-phase cells and repressed in stationary-phase cells, which is consistent with the observed cleavage of Sup35; the cleavage occurs in log-phase cells and is repressed in stationary-phase cells. In this regard, we have confirmed that bafilomycin A1, the specific V-ATPase inhibitor that makes the cytosolic pH acidic, inhibited the cleavage of Sup35 (supplemental Fig. S4). These data suggest the possibility that the changes in the intracellular pH by extracellular glucose may regulate the pH-dependent PrB activity and resultant cleavage of Sup35. Rates of spontaneous *de novo* [*PSI*$^+$] formation are typically low, at a level of 10^{-6} (32, 33). This is much lower than those for some other prions, *e.g.* [*PIN*$^+$] (3). The [*PSI*$^+$] prion is rare in wild and industrial yeast isolates: a recent study detected it in only 10 of 690 strains analyzed (34). In the same study, at least about ⅓ of the tested strains contained some other prions. These data suggest that yeast must have evolved mechanisms to suppress the formation of [*PSI*$^+$]. Our results presented here potentially uncover one such mechanism. In humans, prion diseases such as Creutzfeldt–Jakob disease are caused by converting the cellular prion protein (PrP^c) to its pathogenic and β-sheet-rich isoform (PrP^{sc}) (35, 36). The α-cleavage of PrP^c occurs within the neurotoxic domain, which is essential for the conformational conversion to PrP^{sc} (37, 38), and removes the N-terminal polybasic region, which is important for the initial interaction between PrP^c and PrP^{sc} in the process of misfolding (39, 40). This indicates that the α-cleavage of PrP^c might be a defense mechanism against human prion propagation. Another example is Alzheimer's disease, which is

thought to be caused by the accumulation of amyloid β (Aβ) peptide derived from the proteolytic cleavage of amyloid precursor protein (APP). An alternative cleavage of APP by α-secretase destroys the Aβ sequence and inhibits Aβ production (41). As a result, α-secretase cleavage of APP is protective against Alzheimer's disease. Thus, the proteolytic cleavage of amyloidogenic proteins might be used as a defense mechanism against amyloidosis, in common from



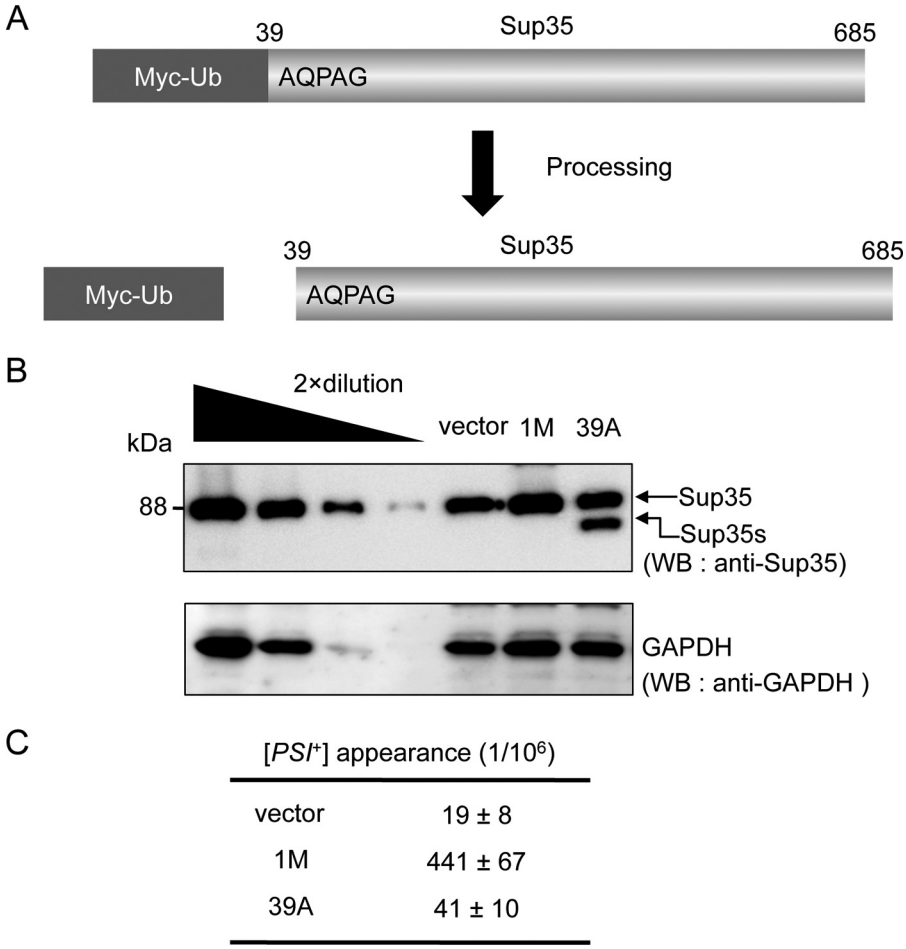


Figure 6. Sup35s itself does not suppress *de novo* prion generation. *A,* schematic representation of the Ub-tagged Sup35 deleted of the amino-terminal 38 amino acids and its processing to 39ASup35. *B,* wild-type (yAO90), YCplac22-Myc-Ub-SUP35(1M) transformants (yAO91), and YCplac22-Myc-Ub-SUP35(39A) transformants (yAO92) were grown in YPDA and harvested at the early log-phase. Cell extracts were analyzed by Western blotting (*WB*) using anti-Sup35 and GAPDH. The *four leftmost lanes* indicate 2-fold dilutions of the protein to confirm that the condition used was semiquantitative. *C,* the frequency of the formation of [*PSI*$^+$] in wild-type, Myc-Ub-SUP35(1M) transformants, and Myc-Ub-SUP35(39A) transformants was calculated as described in the legend to Fig. 5*B*. Guanidine curability of strains is checked and the significant proportion of them is confirmed to be curable (supplemental Table S3). Average results of 3 repeats are shown, *bars* correspond to standard deviations.

yeast to humans. By uncovering the mechanism that suppresses prion generation in yeast, our study may also shed light on defense mechanisms against prion diseases, as well as noninfectious amyloidoses in higher eukaryotes including humans.

Experimental procedures

Yeast strains and media

All *Saccharomyces cerevisiae* strains used in this study are listed in supplemental Table S1. The yeast knockout collection was purchased from EUROSCARF (16). GT17, OT60, OT55, and OT56, which are derived from 74D-694, were previously described (42, 43). All strains were grown at 30 °C either in YPDA (1% yeast extract, 2% peptone, 2% glucose, and 100 μg/ml of adenine) or defined minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate amino acids. To select the KanMX, cells were grown in YPDA medium containing 200 μg/ml of G418. Yeast strains were grown on YPD solid medium containing a low concentration adenine (1% yeast extract, 2% peptone, 2% glucose, 10 μg/ml adenine, 2% agar) for yeast color selection.

Figure 5. The PrA–PrB proteolytic pathway negatively regulates *de novo* formation of [*PSI*$^+$]. *A,* wild-type (OT60) grown in YPDA were harvested at the early log-phase or the late log-phase. Cell extracts were analyzed by Western blotting (*WB*) using anti-Sup35 or anti-PrB. *B,* cells cultured as in *A* were plated onto an adenine-deficient SD plates (*SD-Ade*). Colonies grown on the SD-Ade plates were tested for curability on YPD plates containing 2.5 mM guanidine HCl; numbers of Ade$^+$ colonies cured by guanidine HCl were used to calculate the frequencies of spontaneous [*PSI*$^+$] formation. Average results of 3 repeats are shown, *bars* correspond to standard deviations. *C,* wild-type (OT60) and *pep4*Δ (yAO121) grown in YPDA were harvested at the late log-phase. Cell extracts were analyzed as in *A*. *D,* the frequencies of [*PSI*$^+$] formation in wild-type and *pep4*Δ in *C* were measured as in *B*. *E,* wild-type (OT60) and *prb1*Δ (yAO66) grown in YPDA were harvested at the late log-phase. Cell extracts were analyzed as in *A*. *F,* the frequency of the appearance of [*PSI*$^+$] in wild-type or *pep4*Δ in *E* was calculated as in *B*. *G,* wild-type (OT60) and *prc1*Δ (yAO179) grown in YPDA were harvested at the late log-phase. Cell extracts were analyzed as in *A*. *H,* the frequency of the appearance of [*PSI*$^+$] in wild-type or *pep4*Δ in *G* was calculated as in *B*. *I,* wild-type (yAO109) and p8L2-PRB1 transformants (yAO110) grown in YPDA were harvested at the early log-phase. Cell extracts were analyzed as in *A*. *J,* the frequency of the appearance of [*PSI*$^+$] in wild-type or p8L2-PRB1 transformants was calculated as in *B*. Guanidine curability of all strains in *B*, *D*, *F*, *H*, and *J* is shown in supplemental Table S3. Average results of 3 repeats are shown, *bars* correspond to standard deviations.

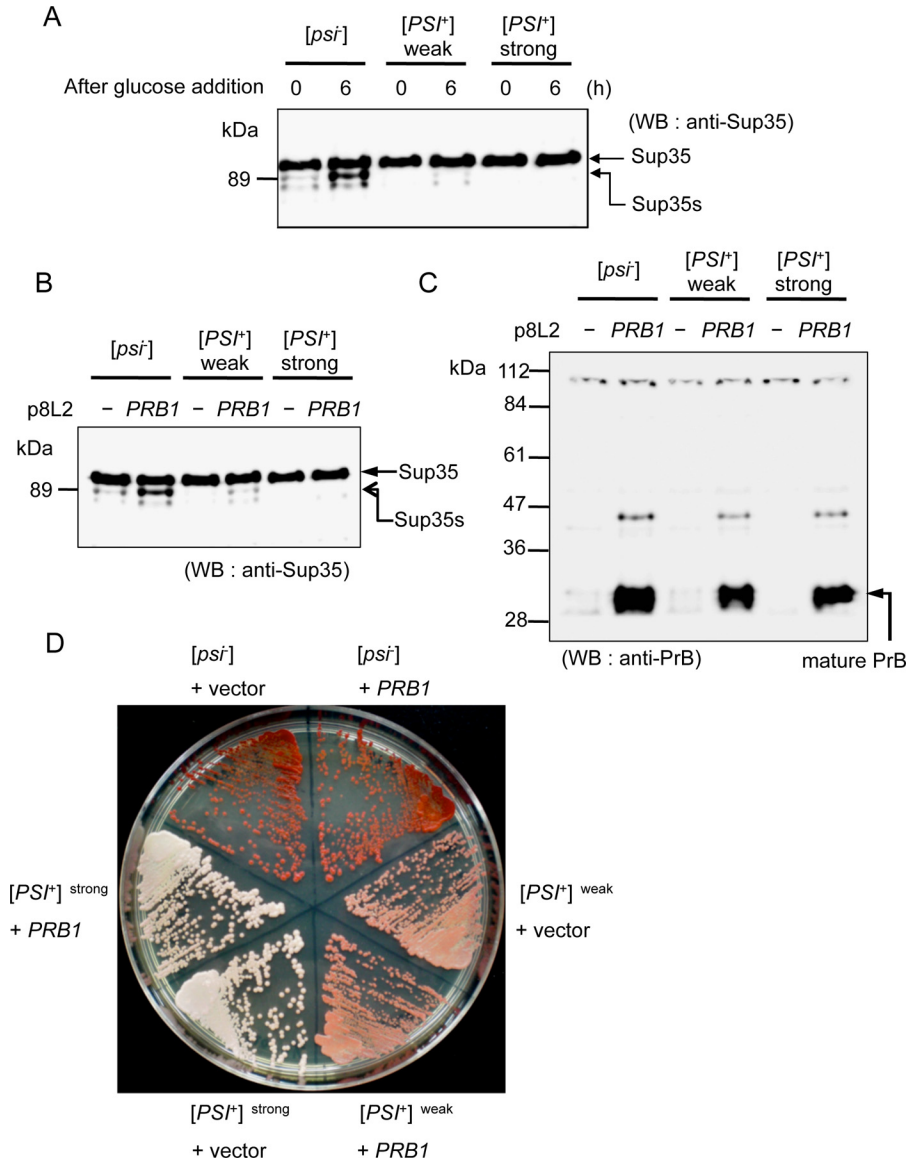


Figure 7. PrB activation neither cleaves the amyloid conformers of Sup35 nor eliminates preexisting [*PSI*$^+$]. *A,* glucose-starved [*psi*$^-$], [*PSI*$^+$]^{weak}, and [*PSI*$^+$]^{strong} cells were cultured in YPDA medium, harvested at the specified time points, and analyzed by Western blotting (*WB*) using anti-Sup35. *B,* [*psi*$^-$], [*PSI*$^+$]^{weak}, or [*PSI*$^+$]^{strong} cells transformed with either p8L2-Flag or p8L2-PRB1 were grown in YPDA and harvested at the early log-phase. Cells extracts were analyzed by Western blotting using anti-Sup35. *C,* cells extracts in *B* were analyzed using anti-PrB. *D,* cells as in *B* were streaked on YPD plates to evaluate if *PRB1* transformants were [*PSI*$^+$] or [*psi*$^-$] (see "Experimental procedures"). *White* and *pink* colonies represent strong and weak [*PSI*$^+$], respectively. Mitotic stability of these plasmids is shown in supplemental Table S4.

Yeast construction and genetic manipulation

SUP35 gene disruption was performed in the OT60 strain, which had been transformed with pKT10-SUP35C (44). The targeting construct, which contained *Candida glabrata HIS3* selection marker (*CgHIS3*), was generated by PCR using pBS-CgHIS3 (45) and the primer pairs AO0009/AO0010. The *SUP35*-disrupted strain was further transformed with YCplac22-SUP35-HA, and pKT10-SUP35C was eliminated by negative selection using 5-fluoroorotic acid (46). For disruption of *PRB1*, *PEP4*, and *PRC1* genes, the targeting constructs were generated as described above using the primer pairs AO0036/AO0037, AO0069/AO0070, and AO0102/AO0103, respectively. The synthetic oligonucleotides used in this study are listed in supplemental Table S2.

Plasmid construction

YCplac22-Flag-SUP35(WT) and YCplac22-SUP35(WT)-Flag were constructed by inverse PCR using YCplac-SUP35 (44) and the primer pairs AO0082/AO0083 and AO0080/AO0081, respectively. YCplac22-Myc-Ub-SUP35(1M) and YCplac22-Myc-Ub-SUP35(39A) were constructed by inverse PCR using YCplac22-Myc-Ub-SUP35(ATG BamHI) and the primer pairs AO0025/AO0020 and AO0025/AO0022, respectively. For creating YCplac22-Myc-Ub-SUP35(ATG BamHI), YCplac22-SUP35 (ATG BamHI) was first prepared by inverse PCR using YCplac22-SUP35(WT) and the primer pair AO0015/AO0016. Myc-Ub fragment was then PCR-amplified using pCMV-Myc-Flag-Ub-GSPT1 (47) and primer pair AO0018/AO0019, and inserted into the BamHI site of YCplac22-SUP35(ATG

BamHI). To construct the multicopy vector p9L2, the *TDH3* terminator sequence was PCR-amplified using pKT10 (44) and the primer pair NH0209/NH0210, and inserted into the BglII and PstI sites of p9L (45). The p9L2-FLAG, which contained the Kozak and FLAG tag sequences, was generated by inverse PCR using p9L2 and the primer pair NH0206/NH0207. To construct the multicopy vector p9L2-FLAG-PRB1, the *PRB1* gene was PCR-amplified using genomic DNA from the GT17 strain and the primer pair AO0051/AO0052, and inserted into the BglII site of the p9L2-FLAG. To construct the single copy vector p8L, the cassette harboring the *tetO* promoter and *CYC1* terminator was excised from pCM190 and inserted into YCplac111. To construct the single copy vector p8L2-FLAG, the Kozak-FLAG-*TDH3* terminator was PCR-amplified using p9L2-FLAG and the primer pair AO0086/AO0087, and inserted into the BamHI site of p8L. To construct the single copy vector p8L2-FLAG-PRB1, the Kozak-FLAG-*PRB1-TDH3* terminator fragment was PCR-amplified using p9L2-FLAG-PRB1 and the primer pair AO0086/AO0087, and inserted into the BamHI site of the p8L. The p8L2-PRB1 was constructed by inverse PCR using p8L2-FLAG-PRB1 and the primer pair AO0073/AO0074. p8L2-PRB1-FLAG was constructed by inverse PCR using p8L2-PRB1 and the primer pair AO0119/AO0120. The single copy vector YCplac22-SUP35-HA was constructed by inverse PCR using YCplac-SUP35 (44) and the primer pair FN01/FN02. The synthetic oligonucleotides used in this study are listed in supplemental Table S2.

Analysis of the proteolytic cleavage of Sup35

Yeast cells were grown to an A_{600} of 2 (mid log-phase) in YPDA medium, and cells were collected and resuspended in medium lacking a carbon source (YPA). After cells were cultured for 12 h in YPA, cells were collected and resuspended in YPDA with the indicated reagents. The cells were further incubated for the specified times and analyzed by Western blotting.

Preparation of yeast extracts and detection of yeast proteins

Yeast extracts were prepared as previously described (48). The extracts were analyzed by Western blotting using the following antibodies; anti-Sup35 antibody raised against His-tagged Sup35 (136–483), anti-Prb1 antibody raised against His-tagged mature Prb1 (281–573), anti-GAPDH antibody raised against His-tagged human GAPDH (1–335), and anti-FLAG M2 antibody (Sigma).

PrB purification and in vitro Sup35 cleavage reaction

Freshly produced colonies of yeast cultures (yAO226, yAO227, and yKO5) were inoculated into selective medium and incubated for 14 h. The culture was diluted to A_{600} of 0.3 in YPDA medium and grown for 24 h to an A_{600} of ~8 (stationary phase). Cells were collected by centrifugation, resuspended either in lysis buffer A (100 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl) for yAO226 and yAO227 strains or in extraction buffer (100 mM Tris-maleate (pH 5.0 or 7.0)) for the yKO5 strain. Proteins were extracted by seven cycles of vortexing with zirconia beads using beads crasher μT-12 (TAITEC). After centrifugation at 20,400 × *g* for 15 min, the supernatants were used as the yeast extract. The extracts isolated from yAO226 and yAO227 strains, which were supplemented with 0.1% of Nonidet P-40, were mixed with anti-FLAG M2 Affinity Gel (Sigma) and were further incubated at 4 °C for 2 h. The beads were washed three times with wash buffer A (100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.1% Nonidet P-40). Sup35 cleavage reaction was performed by incubating the FLAG-PrB bound beads with yeast cell extract from yKO5 strain at 30 °C with shaking. An aliquot of the reaction mixture was collected at the indicated time points and analyzed by Western blotting. For the azocoll assay, the FLAG-PrB bound beads were incubated in reaction buffer (100 mM Tris-maleate (pH 5.0–8.5), 0.05% SDS, 10 mg/ml of azocoll) at 30 °C for 15 min with shaking. PrB activity was calculated by measuring absorbance at 520 nm (27).

Scoring the frequency of spontaneous [PSI$^+$] formation

The phenotypic detection of yeast [*PSI*$^+$] was performed as described (49). Cultures of wild-type (OT60 strain) and knock-out strains derived from OT60 were grown to an A_{600} of ~5 (late log-phase) in 1 ml of YPDA, and each culture was diluted into 5 ml of YPDA at an A_{600} of ~0.3 and grown for a further 6 h to an A_{600} of ~1 (early log-phase, p8L2-PRB1 transformant) or 10 h to an A_{600} of ~5 (late log-phase, serine protease knock-out strains). Each culture (total of ~1×10^6 cells/plate, 10 plates) was plated on adenine-deficient SD plates (SD-Ade) to score for the frequency of spontaneous [*PSI*$^+$] appearance. A 1000-fold dilution of the culture (total of ~200 cells/plate, 3 plates) was plated on YPD plates containing a low concentration of adenine (10 μg/ml) (YPD) to assess living cells. YPD plates were incubated for 3 days, and SD-Ade plates were incubated for 10 days. Colonies on the SD-Ade plates were picked up and streaked on YPD plates and incubated for 2–3 days to recheck [*PSI*$^+$] appearance. We did not detect red colonies on these plates. The streaked colonies were passed to YPD plates containing 2.5 mM guanidine HCl and grown for 2–3 days. The passage was repeated three times. The colonies that produced red subcolonies on YPD plate were determined to be curable.

Affinity purification of cleaved Sup35

The yeast culture (yAO53) grown to an A_{600} of ~2 (mid log-phase) was collected and resuspended in YPA and grown for 12 h. The cells were further grown in YPDA medium for 10 h, and then treated with 100 μg/ml of CHX for 15 min. The yeast cells were washed once with water containing 100 μg/ml of CHX and resuspended in lysis buffer B (20 mM HEPES-KOH (pH 7.5), 5 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 1 mM PMSF, 2 μg/ml of aprotinin, 100 μg/ml of CHX, and protease inhibitor mixture (Roche Applied Science)). Proteins were extracted using beads crasher as described above. The extract, which was supplemented with 1% Nonidet P-40, was incubated with Anti-HA Affinity Matrix (Roche Applied Science) at 4 °C for 3 h. The resin was washed five times with wash buffer B (20 mM HEPES-KOH (pH 7.5), 5 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 100 μg/ml of CHX, and 1% Nonidet P-40). The proteins retained in the resin were eluted with an SDS-PAGE sample buffer by boiling for 10 min. The eluted proteins were separated by SDS-PAGE and transferred to Immobilon-P (Mil-

lipore). The cleaved Sup35 retained on the membrane was analyzed using N-terminal Edman degradation.

Polysome profile analysis

The yeast culture (wild-type (OT60) and *prb1*Δ (yAO66)) grown to an A_{600} of ~2 (mid log-phase) was collected and resuspended in YPA and grown for 12 h. Cells were collected, further grown for 11 h in YPDA, and then treated with 100 μg/ml of CHX for 5 min. The yeast culture was washed once with water containing 100 μg/ml of CHX and resuspended in lysis buffer C (20 mM HEPES-KOH (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10 mM KCl, 0.1 mM PMSF, 100 μg/ml of CHX, and protease inhibitor mixture (Roche Applied Science)). Proteins were extracted using beads crasher as described above. The equivalent of 20 A_{260} units of the extract was then layered on linear 10- 50% sucrose density gradients and centrifuged at 110,500 × *g* for 100 min at 4 °C. Gradients were then fractionated using piston gradient fractionator (BIOCOMP). The distribution of polysome was monitored by continuous absorbance measurement at 254 nm. Proteins in the fractions were precipitated with trichloroacetic acid and then resuspended in HU buffer (200 mM Tris-HCl (pH 8.6), 8 M urea, 5% SDS, 1 mM EDTA, 0.002% bromphenol blue). The samples were analyzed by Western blotting.

Analysis of mitotic stability of plasmid

The [*psi*$^-$], [*PSI*$^+$]^{weak}, and [*PSI*$^+$]^{strong} strains transformed with either p8L2-FLAG or p8L2-PRB1 at an A_{600} of ~1 (early log-phase) in YPDA medium were diluted and plated on the selective plates or YPD containing low adenine plates. The ratio of the number of colonies on the selective plate to that on the YPD containing low adenine plate was calculated to determine the percentage of cells carrying plasmids.

Author contributions—A. O., N. H., Y. O. C., and S. H. designed the experiments; A. O., N. H., A. T., and G. P. N. performed the experiments; A. O., G. P. N., N. H., Y. O. C., and S. H. analyzed the data; A. O., Y. O. C., and S. H. wrote the paper; S. H. conceived and directed the study.

References

1. Wickner, R. B. (2012) Discovering protein-based inheritance through yeast genetics. *J. Biol. Chem.* **287,** 14432–14442
2. Liebman, S. W., and Chernoff, Y. O. (2012) Prions in yeast. *Genetics* **191,** 1041–1072
3. Tuite, M. F., and Serio, T. R. (2010) The prion hypothesis: from biological anomaly to basic regulatory mechanism. *Nat. Rev. Mol. Cell Biol.* **11,** 823–833
4. Shorter, J., and Lindquist, S. (2005) Prions as adaptive conduits of memory and inheritance. *Nat. Rev. Genet.* **6,** 435–450
5. Weissmann, C. (2004) The state of the prion. *Nat. Rev. Microbiol.* **2,** 861–871
6. Ter-Avanesyan, M. D., Kushnirov, V. V., Dagkesamanskaya, A. R., Didichenko, S. A., Chernoff, Y. O., Inge-Vechtomov, S. G., and Smirnov, V. N. (1993) Deletion analysis of the SUP35 gene of the yeast *Saccharomyces cerevisiae* reveals two non-overlapping functional regions in the encoded protein. *Mol. Microbiol.* **7,** 683–692
7. Derkatch, I. L., Chernoff, Y. O., Kushnirov, V. V., Inge-Vechtomov, S. G., and Liebman, S. W. (1996) Genesis and variability of [PSI] prion factors in *Saccharomyces cerevisiae*. *Genetics* **144,** 1375–1386
8. Patino, M. M., Liu, J. J., Glover, J. R., and Lindquist, S. (1996) Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. *Science* **273,** 622–626
9. Doel, S. M., McCready, S. J., Nierras, C. R., and Cox, B. S. (1994) The dominant PNM2- mutation which eliminates the psi factor of *Saccharomyces cerevisiae* is the result of a missense mutation in the *SUP35* gene. *Genetics* **137,** 659–670
10. Ter-Avanesyan, M. D., Dagkesamanskaya, A. R., Kushnirov, V. V., and Smirnov, V. N. (1994) The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast *Saccharomyces cerevisiae*. *Genetics* **137,** 671–676
11. Osherovich, L. Z., Cox, B. S., Tuite, M. F., and Weissman, J. S. (2004) Dissection and design of yeast prions. *PLos Biol.* **2,** E86
12. Borchsenius, A. S., Wegrzyn, R. D., Newnam, G. P., Inge-Vechtomov, S. G., and Chernoff, Y. O. (2001) Yeast prion protein derivative defective in aggregate shearing and production of new "seeds". *EMBO J.* **20,** 6683–6691
13. Ganusova, E. E., Ozolins, L. N., Bhagat, S., Newnam, G. P., Wegrzyn, R. D., Sherman, M. Y., and Chernoff, Y. O. (2006) Modulation of prion formation, aggregation, and toxicity by the actin cytoskeleton in yeast. *Mol. Cell. Biol.* **26,** 617–629
14. Chernova, T. A., Romanyuk, A. V., Karpova, T. S., Shanks, J. R., Ali, M., Moffatt, N., Howie, R. L., O'Dell, A., McNally, J. G., Liebman, S. W., Chernoff, Y. O., and Wilkinson, K. D. (2011) Prion induction by the short-lived, stress-induced protein Lsb2 is regulated by ubiquitination and association with the actin cytoskeleton. *Mol. Cell* **43,** 242–252
15. Manogaran, A. L., Hong, J. Y., Hufana, J., Tyedmers, J., Lindquist, S., and Liebman, S. W. (2011) Prion formation and polyglutamine aggregation are controlled by two classes of genes. *PLoS Genet.* **7,** e1001386
16. Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., *et al.* (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. *Science* **285,** 901–906
17. Moehle, C. M., Tizard, R., Lemmon, S. K., Smart, J., and Jones, E. W. (1987) Protease B of the lysosomelike vacuole of the yeast *Saccharomyces cerevisiae* is homologous to the subtilisin family of serine proteases. *Mol. Cell. Biol.* **7,** 4390–4399
18. Nebes, V. L., and Jones, E. W. (1991) Activation of the proteinase B precursor of the yeast *Saccharomyces cerevisiae* by autocatalysis and by an internal sequence. *J. Biol. Chem.* **266,** 22851–22857
19. Xue, Y., Vashisht, A. A., Tan, Y., Su, T., and Wohlschlegel, J. A. (2014) PRB1 is required for clipping of the histone H3 N terminal tail in *Saccharomyces cerevisiae*. *PLoS ONE* **9,** e90496
20. Derkatch, I. L., Bradley, M. E., Zhou, P., Chernoff, Y. O., and Liebman, S. W. (1997) Genetic and environmental factors affecting the *de novo* appearance of the [PSI$^+$] prion in *Saccharomyces cerevisiae*. *Genetics* **147,** 507–519
21. Chernoff, Y. O., Derkach, I. L., and Inge-Vechtomov, S. G. (1993) Multicopy *SUP35* gene induces *de novo* appearance of psi-like factors in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **24,** 268–270
22. Zhou, P., Derkatch, I. L., Uptain, S. M., Patino, M. M., Lindquist, S., and Liebman, S. W. (1999) The yeast non-Mendelian factor [ETA$^+$] is a variant of [PSI$^+$], a prion-like form of release factor eRF3. *EMBO J.* **18,** 1182–1191
23. Chernoff, Y. O., Uptain, S. M., and Lindquist, S. L. (2002) Analysis of prion factors in yeast. *Methods Enzymol.* **351,** 499–538
24. Paushkin, S. V., Kushnirov, V. V., Smirnov, V. N., and Ter-Avanesyan, M. D. (1996) Propagation of the yeast prion-like [*psi*$^+$] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor. *EMBO J.* **15,** 3127–3134
25. Brett, C. L., Kallay, L., Hua, Z., Green, R., Chyou, A., Zhang, Y., Graham, T. R., Donowitz, M., and Rao, R. (2011) Genome-wide analysis reveals the vacuolar pH-stat of *Saccharomyces cerevisiae*. *PLoS ONE* **6,** e17619
26. Mechler, B., Müller, H., and Wolf, D. H. (1987) Maturation of vacuolar (lysosomal) enzymes in yeast: proteinase yscA and proteinase yscB are catalysts of the processing and activation event of carboxypeptidase yscY. *EMBO J.* **6,** 2157–2163

27. Jones, E. W. (2002) Vacuolar proteases and proteolytic artifacts in *Saccharomyces cerevisiae*. *Methods Enzymol.* **351,** 127–150
28. Moehle, C. M., Aynardi, M. W., Kolodny, M. R., Park, F. J., and Jones, E. W. (1987) Protease B of *Saccharomyces cerevisiae*: isolation and regulation of the PRB1 structural gene. *Genetics* **115,** 255–263
29. Moehle, C. M., and Jones, E. W. (1990) Consequences of growth media, gene copy number, and regulatory mutations on the expression of the *PRB1* gene of *Saccharomyces cerevisiae*. *Genetics* **124,** 39–55
30. Dechant, R., Binda, M., Lee, S. S., Pelet, S., Winderickx, J., and Peter, M. (2010) Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase. *EMBO J.* **29,** 2515–2526
31. Orij, R., Urbanus, M. L., Vizeacoumar, F. J., Giaever, G., Boone, C., Nislow, C., Brul, S., and Smits, G. J. (2012) Genome-wide analysis of intracellular pH reveals quantitative control of cell division rate by pH(c) in *Saccharomyces cerevisiae*. *Genome Biol* **13,** R80
32. Chernoff, Y. O., Newnam, G. P., Kumar, J., Allen, K., and Zink, A. D. (1999) Evidence for a protein mutator in yeast: role of the Hsp70-related chaperone ssb in formation, stability, and toxicity of the [PSI] prion. *Mol. Cell. Biol.* **19,** 8103–8112
33. Lancaster, A. K., Bardill, J. P., True, H. L., and Masel, J. (2010) The spontaneous appearance rate of the yeast prion [PSI^+] and its implications for the evolution of the evolvability properties of the [PSI^+] system. *Genetics* **184,** 393–400
34. Halfmann, R., Jarosz, D. F., Jones, S. K., Chang, A., Lancaster, A. K., and Lindquist, S. (2012) Prions are a common mechanism for phenotypic inheritance in wild yeasts. *Nature* **482,** 363–368
35. Prusiner, S. B., Scott, M. R., DeArmond, S. J., and Cohen, F. E. (1998) Prion protein biology. *Cell* **93,** 337–348
36. Geissen, M., Krasemann, S., Matschke, J., and Glatzel, M. (2007) Understanding the natural variability of prion diseases. *Vaccine* **25,** 5631–5636
37. Chen, S. G., Teplow, D. B., Parchi, P., Teller, J. K., Gambetti, P., and Autilio-Gambetti, L. (1995) Truncated forms of the human prion protein in normal brain and in prion diseases. *J. Biol. Chem.* **270,** 19173–19180
38. Gasset, M., Baldwin, M. A., Lloyd, D. H., Gabriel, J. M., Holtzman, D. M., Cohen, F., Fletterick, R., and Prusiner, S. B. (1992) Predicted α-helical regions of the prion protein when synthesized as peptides form amyloid. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10940–10944
39. Solforosi, L., Bellon, A., Schaller, M., Cruite, J. T., Abalos, G. C., and Williamson, R. A. (2007) Toward molecular dissection of PrPC–PrPSc interactions. *J. Biol. Chem.* **282,** 7465–7471
40. Turnbaugh, J. A., Unterberger, U., Saá, P., Massignan, T., Fluharty, B. R., Bowman, F. P., Miller, M. B., Supattapone, S., Biasini, E., and Harris, D. A. (2012) The N-terminal, polybasic region of PrP(C) dictates the efficiency of prion propagation by binding to PrP(Sc). *J. Neurosci.* **32,** 8817–8830
41. Chasseigneaux, S., and Allinquant, B. (2012) Functions of Aβ, sAPPα and sAPPβ: similarities and differences. *J. Neurochem.* **120,** 99–108
42. Bailleul, P. A., Newnam, G. P., Steenbergen, J. N., and Chernoff, Y. O. (1999) Genetic study of interactions between the cytoskeletal assembly protein sla1 and prion-forming domain of the release factor Sup35 (eRF3) in *Saccharomyces cerevisiae*. *Genetics* **153,** 81–94
43. Newnam, G. P., Wegrzyn, R. D., Lindquist, S. L., and Chernoff, Y. O. (1999) Antagonistic interactions between yeast chaperones Hsp104 and Hsp70 in prion curing. *Mol. Cell. Biol.* **19,** 1325–1333
44. Hosoda, N., Kobayashi, T., Uchida, N., Funakoshi, Y., Kikuchi, Y., Hoshino, S., and Katada, T. (2003) Translation termination factor eRF3 mediates mRNA decay through the regulation of deadenylation. *J. Biol. Chem.* **278,** 38287–38291
45. Funakoshi, Y., Doi, Y., Hosoda, N., Uchida, N., Osawa, M., Shimada, I., Tsujimoto, M., Suzuki, T., Katada, T., and Hoshino, S. (2007) Mechanism of mRNA deadenylation: evidence for a molecular interplay between translation termination factor eRF3 and mRNA deadenylases. *Genes Dev.* **21,** 3135–3148
46. Boeke, J. D., LaCroute, F., and Fink, G. R. (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197,** 345–346
47. Hashimoto, Y., Hosoda, N., Datta, P., Alnemri, E. S., and Hoshino, S. (2012) Translation termination factor eRF3 is targeted for caspase-mediated proteolytic cleavage and degradation during DNA damage-induced apoptosis. *Apoptosis* **17,** 1287–1299
48. Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K., and Schiebel, E. (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* **15,** 963–972
49. Tuite, M. F., Mundy, C. R., and Cox, B. S. (1981) Agents that cause a high frequency of genetic change from [psi^+] to [psi^-] in *Saccharomyces cerevisiae*. *Genetics* **98,** 691–711