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Prion Induction by the Short-lived Stress Induced Protein Lsb2 Is Regulated by Ubiquitination and Association with the Actin Cytoskeleton

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SUMMARY

Yeast prions are self-perpetuating QN-rich amyloids, that control heritable traits and serve as a model for mammalian amyloidoses. De novo prion formation by overproduced prion protein is facilitated by other aggregated QN-rich protein(s), and is influenced by alterations of protein homeostasis. Here we explore the mechanism by which the Las17-binding protein Lsb2 (Pin3) promotes conversion of the translation termination factor Sup35 into its prion form \[\text{PSI}^+\]. We show that Lsb2 localizes with some Sup35 aggregates and that Lsb2 is a short-lived protein whose levels are controlled via the ubiquitin-proteasome system and are dramatically increased by stress. Loss of Lsb2 decreases stability of \[\text{PSI}^+\] after brief heat shock. Mutations interfering with Lsb2 ubiquitination increase prion induction, while a mutation eliminating association of Lsb2 with the actin cytoskeleton blocks its aggregation and prion–inducing ability. These findings directly implicate the UPS and actin cytoskeleton in regulating prions via a stress-inducible QN-rich protein.

INTRODUCTION

Prions are self-perpetuating amyloid-like protein aggregates that transmit neurodegenerative diseases in mammals and heritable traits in yeast (Prusiner, 1998; Wickner et al., 2008). Most prions are based on highly ordered fibrous cross-β polymers (amyloids), resembling the aggregates involved in other amyloidoses and neural inclusion diseases, such as...
Alzheimer, Parkinson, or Huntington diseases (Chiti and Dobson, 2006). Several phylogenetically unrelated prion proteins have been identified in yeast and other fungi (Alberti et al., 2009; Du et al., 2008; Patel et al., 2009; Wickner et al., 2008). It has been suggested that yeast prions can be rapidly mobilized by unfavorable conditions and may confer an adaptive advantage by acting as “capacitors” of phenotypic variability (True et al., 2004), although this hypothesis is controversial (Nakayashiki et al., 2005).

It remains largely unknown how prions arise in vivo. Yeast prion proteins usually contain QN-rich prion domains (PrDs), responsible for prion propagation. It is possible that the multimerization of a misfolded prion-forming protein results in formation of the initial prion “seed”. This could be facilitated when the prion-forming protein is present at a high local concentration. Indeed, de novo formation of yeast prions is induced by transient overproduction of the prion-forming protein or its PrD (see (Chernoff, 2007; Chernoff et al., 2002; Wickner et al., 2008) for reviews). This process is enhanced by actin cytoskeletal structures that are physically associated with aggregates of some overproduced prion-forming proteins (Ganusova et al., 2006). Conditions known to affect protein levels and homeostasis, such as some physiological stresses or alterations of the ubiquitin-proteasome system (UPS) (Allen et al., 2007; Chernova et al., 2003; Tyedmers et al., 2008), also increase de novo prion formation.

Protein ubiquitination is a reversible post-translational modification in which the ubiquitin (Ub) peptide is covalently linked to lysine residues in target proteins (Hershko and Ciechanover, 1998). Since a major function of ubiquitination is to target misfolded proteins for degradation via the proteasome, prion aggregation could be promoted by stress-induced protein misfolding or impairment of UPS dependent degradation of misfolded proteins (Goldberg, 2003). However despite an extensive search, ubiquitination of yeast prion proteins has not been detected (e.g., see (Allen et al., 2007)).

Non-homologous aggregating proteins of similar amino acid composition also modulate prion formation. For example, de novo induction of [PSI⁺], a prion form of the yeast translation termination factor Sup35, by overproduced Sup35 or its PrD is usually efficient only in the presence of another pre-existing prion, e.g. [PIN⁺] or [RNQ⁺] (Derkatch et al., 1997), a prion form of the Rnq1 protein (Derkatch et al., 2001; Osherovich and Weissman, 2001). Other prions, or other overproduced yeast proteins with QN-rich domains, also promote de novo [PSI⁺] induction in the absence of [RNQ⁺]. It is thought that pre-existing prion aggregates or aggregates of overproduced heterologous proteins may provide initial nucleation centers for Sup35 aggregation (Derkatch et al., 2001; Derkatch et al., 2004) or sequester co-factors that normally antagonize prion formation by Sup35 (Osherovich and Weissman, 2001). Some [PSI⁺]-promoting proteins have also been shown to form prions of their own (Alberti et al., 2009; Du et al., 2008; Patel et al., 2009). However, it is not known if prion formation induced by overproduction of prion-inducing proteins reflects physiologically relevant processes, since homeostatic mechanisms regulating these proteins have not been studied.

We have developed a model to investigate in vivo prion induction by heterologous prion-inducing proteins. Overproduction of Lsb2 (Las17-binding protein 2), also called Pin3 (from “prion induction”), has been reported to promote [PSI⁺] induction by excess Sup35 (Derkatch et al., 2001). Lsb2 is ubiquitinated (Peng et al., 2003) and binds to both Ub (Tong et al., 2002) and the actin assembly protein Las17 (Madania et al., 1999). Here, we demonstrate that Lsb2 is a short-lived protein, whose ubiquitination reduces its abundance and prion inducing ability, and that the prion-inducing effect of Lsb2 strictly depends on its association with the actin cytoskeleton. Absence of Lsb2 destabilizes [PSI⁺] after brief heat...
shock. Our finding that cellular levels of Lsb2 are dramatically increased by thermal stress points to physiological relevance of the observed phenomena.

RESULTS
Promotion of de novo \([PSI^+]\) Induction by Excess Lsb2

Previously, it was shown that overproduction of Lsb2 (Pin3) promotes aggregation of Sup35 or its PrD (N) domain, resulting in their conversion into a prion state \([PSI^+]\) (Derkatch et al., 2001). This occurs in the absence of other pre-existing prions such as \([RNQ^+]\). We have confirmed these observations using simultaneous co-overproduction of Lsb2 and Sup35 and a newly developed “sequential” protocol where Lsb2 is transiently overproduced before Sup35 overproduction. In both cases, \([PSI^+]\) induction was detected by growth on medium lacking adenine (–Ade) after expression of both proteins was turned off (Figure 1A and S1A). The strains used carry a premature stop codon (UGA) in the \(ADE1\) gene (ade1-14 see (Chernoff et al., 2002)) and cannot grow on –Ade media in the presence of functional Sup35. Upon prion formation, a partial loss of Sup35 function allows read through and growth on –Ade medium. The Ade\(^+\) colonies were confirmed to contain the \([PSI^+]\) prion by demonstrating that the Ade\(^+\) phenotype is curable by GuHCl (data not shown) and by overproduction of the chaperone Hsp104 (see below), agents known to eliminate \([PSI^+]\) (Chernoff et al., 2002).

Notably, overproduction of a similar level of Lsb1 (Figure S1B), a close Lsb2 paralog, does not promote \([PSI^+]\) induction by excess Sup35 in the absence of \([RNQ^+]\) (Figure 1B, C). Lsb1 shares 64% amino acid identity with Lsb2 and differs most in the QN-rich region. Shortening the stretch of 8Q residues in Lsb2 to 4Q (as in Lsb1) by substituting Q with A, reduced but did not abolish Lsb2’s ability to promote \([PSI^+]\) induction. Thus, both length of the poly-Q stretch and other Lsb2-specific sequence features are important for this effect.

Lsb2 Forms Punctate Structures, Transiently Associated with Aggregated Sup35

Lsb1 and Lsb2, tagged with green fluorescent protein (GFP) and produced at low levels from the endogenous promoter or \(P_{CUP1}\) promoter in the absence of additional copper, exhibited a weak fluorescence signal evenly distributed throughout the cytoplasm in the majority of cells and was detectable as a single foci in 10-20% of cells, These foci were distinct from P bodies and stress granules, (Figure S1C, D). When expression was increased by excess CuSO\(_4\) both Lsb1-GFP and Lsb2-GFP formed multiple punctate structures. Only puncta formed by Lsb2 displayed polarized localization, highly reminiscent of cortical actin patches in most cells with larger clumps in some cells (Figure 2A and Movie S1).

To determine if Sup35-derived aggregates, generated in the process of prion induction, are associated with structures formed by Lsb2 protein Lsb2-GFP and the Sup35 PrD with middle (M) domain (Sup35NM) tagged with DsRed, were co-expressed at high levels. In agreement with previous observations (Ganusova et al., 2006; Zhou et al., 2001), Sup35NM-DsRed aggregates, induced in the \([psi^-RNQ^+]\) strain, formed ring filaments, clumps and/or dots. Each ring was located at or near a fraction of punctate structures formed by Lsb2-GFP (Movie S2). Remarkably, Sup35 dots were partially overlapping with or fully encircled by Lsb2 puncta (Figure 2B-D). This was a dynamic transient process during which small aggregates of Sup35 fused, grew in size (Figure 2B and Movie S3) and density within an area encircled by Lsb2 puncta (Figure 2C and Movie S4), followed by dissociation of Lsb2 and Sup35 aggregates, resulting in their independent coexistence (Figure 2D and Movie S5). Similar colocalization of Sup35-dsRED and Lsb2-GFP aggregates was observed in \([rnq^-]\) strain (Figure S4D). Also we observed more aggregates in \([RNQ^+]\) (40.2±10.7%) than in
[rnq] (22.3±8.1%, P > 0.0001) strains after induction of the Lsb2-GFP had been stopped for many generations indicating that presence of prion [RNQ+] stabilizes Lsb2.

**Lsb2 Promotes Formation of the [RNQ+] Prion, but not Detectable Lsb2 Prion**

One potential explanation for the [PSI+] inducing effect of Lsb2 could be that Lsb2 itself forms a prion or stimulates prion formation by another QN-rich protein, which then nucleates formation of the Sup35 prion. However, we obtained no evidence for prion formation by Lsb2. Lsb2-GFP formed aggregates of the same shape and frequency regardless of previous Lsb2-GFP induction. Lsb2-GFP aggregated equally in the presence or absence of the Hsp104 chaperone (data not shown), a factor known (Romanova and Chernoff, 2009) to be required for the propagation of most yeast prions.

In another approach we screened 350 independent [PSI+] isolates, induced by simultaneous transient overexpression of Sup35 and HA-tagged Lsb2, for the presence of other prions capable of promoting prion formation by Sup35 (Figure S1E). These isolates were cured of [PSI+] by transient overproduction of the chaperone Hsp104 which eliminates [PSI+] but not the majority of other known yeast prions (Romanova and Chernoff, 2009). Only isolates that had acquired another prion (not curable by excess Hsp104) would be expected to efficiently become [PSI+] upon overexpression of Sup35 (reinduction). The majority (97%) of cured isolates were unable to produce Ade+ colonies after Sup35 overexpression, as is typical of the original [psi- rnq-] strains. This suggests that in most cases, the presence of the transiently formed Lsb2 aggregates, rather than stable prions, is sufficient for Sup35 nucleation. However, about 3% of the derivatives did become [PSI+]-inducible. These derivatives were checked for the presence of amyloid polymers of Lsb2 and Rnq1. Yeast prions can be distinguished by their insolubility in SDS and subsequent inability to enter an SDS-PAGE gel without boiling (Kushnirov et al., 2006). In all isolates, Lsb2 remained soluble, while Rnq1 was in an SDS-insoluble form (Figure 2E). Thus, simultaneous transient overproduction of Lsb2 and Sup35 induced the formation of stable [RNQ+] prions in about 3% of the colonies that acquired [PSI+], even though Rnq1 itself was not overproduced. This is several orders of magnitude higher than the frequency of spontaneous [RNQ+] formation (Derkatch et al., 2000). The possibility that Lsb2 overexpression induces unstable [RNQ+] formation, which in turn facilitates [PSI+] induction, was eliminated by our finding that overexpressed Lsb2 enhances the appearance of [PSI+] even in rnq1Δ strains (Figure 1A). The fact that isolates bearing the prion form of Lsb2 itself were not found indicates that either wild type Lsb2 does not form an SDS-resistant prion at a measurable frequency or that the prion state of Lsb2 is unstable or sensitive to excess Hsp104.

**Lsb2 is a Stress-Inducible Protein Influencing [PSI+] Maintenance**

As artificial overproduction of Lsb2 promotes its aggregation and enables it to enhance the de novo formation of heterologous prions, we asked if endogenous Lsb2 ever reaches similarly high levels in normal yeast cells. Concentrations of endogenous HA-tagged Lsb1 and Lsb2, expressed from the respective chromosomal loci, were examined in cells grown at 25°C and following heat shock at 37°C, 39°C or 42°C (Figures 3A and S2). Indeed, a transient shift to high temperature induced Lsb2, but not Lsb1, (Figure 3A,S2A and S2B) to levels comparable to our artificial overexpression experiments (Figure 3B) and returned to pre-stress levels after 30-40 min at 37°C, 39°C or 42°C. Lsb2 aggregates were observed during heat shock (Movie S6) and we found that short (15-min) overexpression of Lsb2 from P<sub>CUP1</sub> also induced a measurable increase in [PSI+] (Table S1). In order to determine if Lsb2 influences [PSI+] during stress, we compared the effects of heat shock on [PSI+] maintenance in wild-type and lsb2Δ strains. A weak [PSI+] variant is destabilized by short-term mild heat shock at 39°C, leading to formation of a few [psi+] colonies and a larger fraction of mosaic [PSI+]/[psi+] colonies after return to normal temperature and resumption...
of cell division (Figure 3C) (Newnam et al., 2011). Longer incubation at 39°C results in [PSI⁺] recovery. We found that deletion of LSB2 significantly increases heat-induced [PSI⁺] destabilization (Figure 3D), and causes a slight but observable increase in the frequency of completely “cured” ([psi−]) colonies generated after such a treatment (Figure S2C). Neither wild-type nor lsb2J strains exhibited significant cell death at 39°C (data not shown). Our results implicate Lsb2 in prion maintenance during stress and indicate that the observed effects of Lsb2 levels on [PSI⁺] are physiologically relevant.

**Lsb2 is Ubiquitinated at K80 and Stabilized by Proteasome Defects**

Large scale mass spectrometry analysis previously identified Lsb1 and Lsb2 as ubiquitinated proteins, with residues K41 and K79 (Lsb1), and K80 (Lsb2) identified as ubiquitination sites (Peng et al., 2003). To confirm ubiquitination, we expressed HA-tagged Lsb2 at various levels from the P_CUP1 promoter and found that a single band is observed at low expression levels. Additional high MW bands, resembling a ladder of ubiquitinated protein, appeared at high levels of expression in the presence of Myc-Ub (Figure 4A). Immunoprecipitation (IP) with anti-HA antibody (Ab), followed by immunoblotting with anti-Myc Ab, confirmed that these high MW bands indeed represent ubiquitinated Lsb2 (Figure 4B). Furthermore, K80R mutation increased the levels of non-ubiquitinated Lsb2 (Figure 4C) and abolished high MW bands (Figure 4D). We could not detect ubiquitinated K80R Lsb2 by IP (Figure 4B), demonstrating that under normal conditions, K80 is the major Lsb2 ubiquitination site. Note that the accumulation of a ladder of Lsb2 derivatives was also detected after Lsb2 induction by heat shock (Figure 3A). Since the stress-related burst in Lsb2 levels was short-lived, it is likely that excess Lsb2 is ubiquitinated and degraded via the UPS. Indeed, the proteosomal mutation, doa3-1, caused a significant accumulation of Lsb2 (Figure 4E) and increased its half-life from 15 min to over 40 min. (Figure 4F). The overproduction of Lsb2 from CUP1 promoter to the level that causes its aggregation and Sup35 prion induction results in its half-life increasing from 15 to over 90 min (Figure S2D). This observation probably reflects the stabilization of Lsb2 in the form of prion inducing aggregates in a subpopulation of cells during heat shock. Thus, Lsb2 is the first short-lived protein known to induce prions.

**Lsb2 Is Ubiquitinated by Ubc4/Ubc1 and Rsp5**

Ubc1, Ubc4 and Ubc5 are homologous members of the major family of yeast Ub-conjugating (E2) enzymes involved in protein ubiquitination under various conditions, including environmental stress. Deletion of Ubc4 increases the spontaneous de novo formation of [PSI⁺] (Allen et al., 2007), possibly due to reduced ubiquitination and the resultant accumulation of the QN-rich protein. Indeed, deletion of Ubc4 increased the amount of non-ubiquitinated Lsb2 when it was expressed at low levels and decreased the amount of ubiquitinated Lsb2 when it was overproduced (Figure 5A). Interestingly, the percentage of cells with large Lsb2-GFP aggregates was significantly increased in ubc4Δ cells (Figure S3A). We observed similar results in a ubc1Δ strain but, not in a ubc5Δ strain (Figure 5B and data not shown). We were not able to analyze Lsb2 ubiquitination in the double ubc1/4Δ and ubc4/5Δ deletion strains due to extremely poor growth. Our data indicate that either Ubc1 or Ubc4 can act as the E2 in ubiquitination of Lsb2.

The Lsb2 paralog, Lsb1, has previously been identified as a substrate of the E3 Ub-ligase Rsp5 in a global proteomic screen (Gupta et al., 2007). Likewise, we found that ubiquitinated forms of Lsb2 are absent in an rsp5-1 mutant strain (Figure 5C). Lsb2 contains a putative “PY motif” (PPQY₁²⁷) that is frequently present in Rsp5 substrates, is recognized directly by the WW domains of Rsp5 and needed for Rsp5-dependent ubiquitination (Rotin and Kumar, 2009). Alanine substitution of proline 124 and 125 within the “PY motif” abolished Lsb2 ubiquitination (Figure 5D).
Inhibition of Lsb2 Ubiquitination Enhances its Prion-Inducing Ability

Overproduction of K80R Lsb2 (abolishing the site of ubiquitination), or the double P124A, P125A (abolishing the Rsp5 binding site) was two fold more efficient in promoting \( \text{PSI}^+ \) induction than wild type Lsb2 (Figure 5E). The effects of these mutations were more clearly seen when “sequential” induction of Lsb2 and Sup35 (Figure S1, Table S3) was performed. This protocol mimics transient accumulation of endogenous Lsb2 such as that observed upon heat shock. Thus, effect of a transient Lsb2 overproduction on prion formation is stronger if ubiquitination of Lsb2 is impaired.

Lsb1 and Lsb2 Interact with Las17, Ub, and the Prion Domain of Sup35

Global screens implicate Lsb1 and Lsb2 in binding Las17 and Ub (Madania et al., 1999; Tong et al., 2002). We have confirmed this using yeast two-hybrid analysis, and also showed that both proteins interact with full length Sup35 protein and its N-terminal PrD, (Sup35N) (Figure 6A). Mutation of W90 (Lsb1) or 91 (Lsb2) within the SH3 domain to S abolished interaction with Las17 but did not affect interaction with Sup35N (Figure 6A). Interaction with Ub was also abolished in Lsb2-W91S but was only slightly affected in Lsb1-W90S, showing that other residues or domains may contribute to Ub binding by Lsb1. In vitro pull down experiments with purified proteins confirmed that the Lsb2 SH3 domain itself binds to Ub (Figure 6B).

As this conserved W is essential for most other SH3 mediated protein binding (Evangelista et al., 2000), our data indicate that the Lsb proteins bind Las17 via their N-terminal SH3 domains, while interaction with Sup35N involves a different domain (possibly, the QN-rich PrD-like region).

Lsb2 and Lsb1 Are Colocalized with the Cortical Actin Cytoskeleton

The polarized distribution of overexpressed Lsb2 and its binding to Las17 suggest that Lsb2 may localize to the actin cytoskeleton. Consistent with that we found that punctate structures and aggregates of Lsb2 often colocalize with rhodamine-phalloidin stained actin patches in fixed cells (Figure 6C) and with the actin patch marker protein Cap2-RFP in live cells (Figure 6D and Movie S7). The majority of the large aggregates of Lsb1 and Lsb2 were found either at the periphery of the cell or next to a vacuole (Figure 2A-D and Movie S1). Some were juxtanuclear (Figure S4A), but did not colocalize with Spc29-RFP, a component of the spindle pole body (SPB) (Figure S4B). Notably, the formation of large aggregates was an active process, as it was inhibited by the microtubule-depolymerizing drug benomyl (Figure S4C), suggesting that these aggregates could be formed in one of the microtubule-dependent quality control compartments, recently characterized in yeast (Kaganovich et al., 2008; Wang et al., 2009).

The W91S Mutation in Lsb2 Blocks Its Colocalization with Actin, Aggregation and Prion-Inducing Ability

As actin cytoskeletal structures were shown to enhance \( \text{PSI}^+ \) induction by excess Sup35 (Ganusova et al., 2006), we checked if association with the cytoskeleton is important for the prion-inducing properties of Lsb2. The W90S, W91S substitution within SH3 domain of Lsb1 or Lsb2 completely abolished the formation of the punctate structures and aggregates associated with actin patches, leading to a diffuse distribution of green fluorescence throughout the cytoplasm (Figure 6E). Thus, Lsb proteins are associated with cortical actin patches via their SH3 domain, which controls binding to Las17 and ubiquitin. In contrast, Lsb2 mutants defective in covalent ubiquitination (K80R and P124A, P125A) formed punctate structures similar to wild type Lsb2, indicating that covalent ubiquitination of Lsb2 is not required for Lsb2 association with the actin cytoskeleton (Figure S3B). In contrast to
wild type or ubiquitination-defective Lsb2, overproduction of Lsb2 protein bearing the W91S substitution failed to promote \([\text{PSI}^+]\) induction by excess Sup35 (Figure 6F). This was not due to a decrease in Lsb2 abundance, as levels of Lsb2-W91S mutant were similar to the levels of wild type Lsb2 expressed from the same promoter under the same conditions, with only a slight decrease in the ubiquitinated fraction (Figure 6G, Table S2). Thus, the intact SH3 domain is crucial for association with the actin cytoskeleton, aggregation, and prion inducing ability of Lsb2.

**DISCUSSION**

**Protein Homeostasis, Lsb2 Levels and Prions**

Participation of the UPS in protein quality control is essential for both normal protein folding and coping with stress. For instance, UPS failure leads to the accumulation and aggregation of misfolded proteins (Goldberg, 2003). Prion proteins are one class of “misfolded” proteins that are modulated by the UPS and alterations of the UPS increase formation of the \([\text{PSI}^+]\) prion (Allen et al., 2007; Tyedmers et al., 2008). Environmental stresses induce the UPS and may induce (Tyedmers et al., 2008) or destabilize (Newnam et al., 2011) \([\text{PSI}^+]\). However, stress does not induce expression of Sup35, and evidence for the direct ubiquitination of Sup35 is lacking (Allen et al., 2007). Thus UPS and environmental stresses may modulate \([\text{PSI}^+]\) formation via regulation of ancillary factors. As this work makes clear, the effects of stress on prion induction are mediated, in part, by the action of the UPS on Lsb2 amount and/or function.

It has been hypothesized that prions may play a role in protecting proteins from degradation under unfavorable conditions (Chernoff, 2007), and/or in the adaptation of microbial populations to changing environments (True et al., 2004). If so, we might expect stress-dependent prion induction to be a regulated process. Among the proteins whose overproduction promotes prion formation by Sup35, Lsb2 stands out as having the expected properties of a “stress-dependent prion inducer”. First, large-scale analysis indicated that \(\text{LSB2}\) expression is regulated by the stress-inducible transcription factor Hsf1 (Harbison et al., 2004), and Lsb2 mRNA levels are induced 20-30 fold by various stresses (Gasch et al., 2000). Furthermore, we found that Lsb2 protein levels are dramatically increased by thermal stress (Figure 3A), and return to pre-stress conditions if heat stress is prolonged for longer than 30 min. The finding of a ladder of ubiquitinated Lsb2 species, is indicative of Lsb2 degradation via the UPS. Lsb2 has a half-life of 10-15 minutes, and it is ubiquitinated by the E2 enzymes Ubc1/4 and the E3 ligase Rsp5. Both the Lsb2 half-life and its steady state levels are dramatically increased in a proteasomal mutant (Figure 4E, F). Notably, the levels of Lsb2, accumulated during heat stress, are similar to the levels causing prion induction (Figure 3B), and we have shown that transient (rather than continuous) overproduction of Lsb2 is sufficient for \([\text{PSI}^+]\) induction (Figure S1A). Thus, Lsb2 accumulation in response to physiological stresses could be a trigger that induces prions.

Failure of the UPS (e. g. during severe stress) should stabilize Lsb2 levels, thus exacerbating its potential prion-inducing effect. Indeed, we demonstrated that mutation of Lsb2 ubiquitination site caused Lsb2 accumulation (Figure 4C) and increased prion formation (Figure 5E). Lsb2 accumulation in \(\text{ubc4A}\) cells (Figure 5A) may contribute to the ability of \(\text{ubc4A}\) to enhance the spontaneous appearance of \([\text{PSI}^+]\) (Allen et al., 2007), although other factors are certainly involved; \([\text{PSI}^+]\) formation is still increased, albeit to a lower extent, in the double \(\text{ubc4A lsb2A}\) mutant (data not shown). Altogether, our data suggest that effects of fluctuations in Lsb2 levels on prions are physiologically relevant, for example during environmental stress.
The frequency of prion induction is dependent by the rates of de novo prion formation and the loss of prion from \(\text{[PSI}^+\text{]}\) cells. The frequency of prion induction by stress-induced Lsb2 is difficult to address directly, as its rapid degradation in most cells during stress probably confines its prion-inducing capability to only a small fraction of cells retaining Lsb2 in a transiently aggregated state. However, we find that the lack of Lsb2 destabilizes a weak \(\text{[PSI}^+\text{]}\) variant after short term heat shock (Figure 3D), thus confirming the role of stress-induced Lsb2 in prion maintenance under these conditions. In contrast, prion induction is much easier to demonstrate in a population of cells that are all undergoing artificial overproduction during vegetative growth. The effect on prion induction may be general as Lsb2 overexpression promoted the formation of another prion, \(\text{[RNQ}^+\text{]}\), even when the Rnq1 protein was not overproduced. This agrees with previous observations that heterologous prion proteins may nucleate prion formation by each other (Ross et al., 2009).

**Role of Cytoskeletal Localization of Lsb2 in Prion Formation**

The mechanism, by which heterologous prion inducers act at the molecular level has remained unclear. One possibility is that some of these inducers may direct prionogenic proteins to prion formation sites, where assembly of protein aggregates and/or subsequent conversion into prion is facilitated. The actin cytoskeleton plays an important role in trafficking and deposition of damaged and aggregated yeast proteins (Liu et al., 2010). Sup35 is involved in the regulatory network centered on the actin assembly factor Las17 (Toret and Drubin, 2006), and was shown to interact with some cortical actin cytoskeleton components (Bailleul et al., 1999; Ganusova et al., 2006). These interactions are crucial for prion induction by excess Sup35.

Although both Lsb1 and Lsb2 interact with Las17 and form multiple punctate structures or larger aggregates associated with actin patches (Figure 6 A, D), only Lsb2 exhibits cell cycle dependent polarized distribution and induces \(\text{[PSI}^+\text{]}\). Association of Lsb2 with actin patches occurs via the SH3 domain, most likely due to its interaction with Las17 and/or Ub. It is possible that Ub binding via the SH3 domain modulates association of Lsb2 with the actin cytoskeleton under some circumstances, as shown for other cytoskeletal proteins (Stamenova et al., 2007).

Notably, the same W91S mutation that eliminates the cytoskeletal association of Lsb2 affects Lsb2 ability to aggregate and promote \(\text{[PSI}^+\text{]}\) formation (Figure 6F). These data implicate association with actin patches via SH3 in the prion-inducing properties of Lsb2 and support the model (Ganusova et al., 2006) proposing that cortical actin structures serve as prion induction sites.

**Sup35 and Lsb2 Interact and Associate at Cytoskeletal Sites During Prionogenesis**

In a fraction of \(\text{[psi}^-\text{]}\) cells containing another prion (e.g. \(\text{[RNQ}^+\text{]}\)), overproduced Sup35 is assembled into large aggregates. In addition to dots or clumps, that are also detected in prion-containing (\(\text{[PSI}^+\text{]}\)) cells, filamentous (sometimes ring-like) structures were observed that are specific to prion induction and suggested to represent an intermediate form of a prion (Ganusova et al., 2006; Mathur et al., 2010; Tyedmers et al., 2010; Zhou et al., 2001). Some rings are located at the cell periphery and partly colocalized with cortical cytoskeleton structures, while others are located in the cell interior, surrounding the vacuole. It was proposed that peripheral Sup35 rings, assembled with the help of actin associated proteins, then move away from the periphery with components of the endocytic machinery, eventually resulting in the formation of internal perivacuolar rings (Ganusova et al., 2006). Colocalization of Lsb2 with peripheral and perivacuolar rings, and with some dot-like aggregates of Sup35 (Figure 2 B, C, Movie S2) is in agreement with the requirement for cytoskeletal localization of Lsb2 for its prion-inducing capability. In some cases, Lsb2
punctate structures surrounded Sup35 agglomerate (Figure 2C). Time-lapse microscopy (Movie S4) demonstrates that Lsb2 structures are initially concentrated at sites of subsequent accumulation of Sup35 protein. Indeed, this increase in local Sup35 concentration may cause prion formation.

It is an intriguing possibility, supported by frequent perivacuolar localization of Lsb2 aggregates (Figure 2 B-D), that such prion induction sites may coincide with some quality control compartments, e.g. aggresomes or IPOD (Kaganovich et al., 2008; Wang et al., 2009). Time-lapse data indicate that Lsb2 puncta later dissociate from Sup35 aggregates and coexist as separate foci in the same cell (Figure 2D and Movie S5) in agreement with previous observation that the propagation of mature prions does not require other prions or QN-rich protein aggregates (Derkatch et al., 2000). The inability of Lsb1, which also interacts with Sup35 and with the cytoskeleton, to promote prion formation indicates that Lsb2 exercises an active role in this process, possibly via the poly-Q stretch of Lsb2 forming a “polar zipper” (Perutz et al., 1994) similar in structure to the Sup35 prion. Indeed, shortening of this stretch eight Q to four decreases the prion-inducing potential of Lsb2 (Figure 1C). The stabilization of $\text{PSI}^+$ after brief heat shock (Figure 3C) may reflect the sequestration of Sup35 by binding to the Q/N rich C-terminus of Lsb2.

**Model for the Effect of Lsb2 on prions**

Our model for the role of Lsb2 as a stress dependent prion inducer is shown in Figure 7. We propose that dramatic increase in levels of Lsb2, caused by heat shock and other stresses, triggers the accumulation of misfolded Sup35 at the cytoskeleton-associated cortical locations. Cytoskeletal networks would then target aggregated Sup35 to the quality control compartments, adjacent to the vacuole. This partly protects $\text{PSI}^+$ prion from uncontrolled agglomeration and elimination during short term heat shock. In $\text{psi}^-$ cells, the increased local concentration of Sup35 could facilitate prion formation. This effect probably becomes more pronounced in a small fraction of cells that have accumulated Lsb2 in a transiently aggregated state for longer periods of time. At the end, proteolytically stable prion aggregates could escape the quality control compartments while Lsb2 could be recycled or degraded. Other heterologous inducers might work by a similar mechanism, although not necessarily during stress.

Notably, Lsb2 is not among those yeast proteins shown to form a prion in vivo (Alberti et al., 2009) and our data see above). This indicates that conversion of a heterologous inducer into a prion form may not be a requirement for the prion-inducing effect on another protein. Since Lsb2 is a short-lived protein, it is possible that it forms a very transient prion-like state. It is an attractive possibility that such a short-lived prion-like state of Lsb2 is needed to restrict its ability to target misfolded proteins to the quality control compartments, or serve as prion induction sites after stress conditions are relieved. It is however technically challenging to prove the existence of such a state. Taken together, our results present strong evidence for the ability of UPS and actin cytoskeleton to regulate induction of prions by modulating homeostasis and cellular location of heterologous proteins. As both systems are ubiquitous throughout the eukaryotic kingdom, these data provide a new perspective for understanding protein-based inheritance and triggering mechanisms of protein assembly diseases.
EXPERIMENTAL PROCEDURES

Strains, Growth Conditions

*Saccharomyces cerevisiae* strains and plasmids used in this study are described in Supplemental information. Standard media and growth conditions were used (Sherman, 2002) with incubation at 30°C unless otherwise specified.

Plate Assay for [PSI] Induction

The presence of [PSI] was monitored by its ability to suppress the reporter ade1-14 (UGA), resulting in growth on media lacking adenine (-Ade) (Chernoff et al., 2002). Individual transformants containing plasmids with SUP35 under P<sub>GAL1</sub> and LSB2 under P<sub>CUP1</sub> promoters were patched on synthetic medium (SD) selective for both plasmids and replica plated either onto galactose (Gal) medium with CuSO<sub>4</sub> (Cu) for simultaneous induction of both proteins, or onto SD+Cu medium first (where only Lsb2 is overproduced). After growth for 2 days colonies were replica plated onto Gal medium (where only Sup35 is overproduced) or Gal+Cu (where both proteins are overproduced) for sequential induction. After 4 days of growth on Gal+Cu or Gal, cells were replica plated onto -Ade for [PSI] detection. At least 40 independent transformants were tested for each strain-plasmid combination; the majority (or all) showed the same result in each case.

Protein Analysis

For protein isolation, cells were lysed by boiling in SDS-containing loading buffer, or by vortexing with glass beads (in case of gel entry assay or IP) (Allen et al., 2007). The IP experiments were performed using immobilized protein A (Invitrogen). Cycloheximide chase experiments were performed as described (Katzmann and Wendland, 2005). Protein extracts and immunoprecipitates were examined by Western analysis using specific antibodies, described in the Supplemental information.

Heat Shock Experiments

Exponential yeast cultures, expressing either Lsb1-HA or Lsb2-HA from the endogenous promoter on the chromosome and grown at 25°C, were shifted to 39°C. Aliquots were taken after specified periods of time, cells were lysed by boiling analyzed by Western blotting. For [PSI] curing experiments, aliquots were plated onto YPD medium and incubated at 25°C (Newnan et al. 2011). [PSI] (light pink), [psi] (red) and mosaic [PSI]/[psi] colonies were detected by visual inspection.

Fluorescence Microscopy

Proteins with indicated fluorescent tag were imaged in living cells with a 100X oil immersion objective on the Olympus BX60 microscope (Olympus America, Inc. Melville, NY), equipped with a Quantrix digital camera (Photometrics/Roper Scientific, Tucson, AZ).

Time-lapse microscopy of live cells (Figure 2, 6, S4 and Movies S1-S7) was performed on the LSM510 confocal microscope (Carl Zeiss, Inc., New York, NY), with a 100x 1.35 NA objective and laser lines 543 and 488 see details in supplementary material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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HIGHLIGHTS

- High levels of Lsb2/Pin3 induce formation of both Sup35 and Rnq1 prions.
- Lsb2 is a short-lived protein that is dramatically increased by thermal stress.
- Loss of Lsb2 decreases the stability of the Sup35 prion after brief heat shock.
- Preventing Lsb2 ubiquitination or actin association modulates prion induction.
Figure 1. Homologous Proteins Lsb2 and Lsb1 Differ in Prion-Inducing Ability

(A) Overexpression of Lsb2 enables excess Sup35 to induce $[PSI^{+}]$ in $[\text{rnq}^{-}]$, $\text{rnq1} \Delta$ strains, as detected by growth on –Ade medium. A $[\text{RNQ}^{+}]$ strain is shown as a positive control. See also Figure S1. (B) Structural organization of the Lsb1 and Lsb2 proteins. Black bars and arrow represent conserved lysine (K) and tryptophan (W) residues, respectively. Superscript and subscript numbers respectively correspond to amino acid positions and the number of repeated residues in a stretch, respectively. (C) Poly-Q stretch length influences the $[PSI^{+}]$ promoting capabilities of Lsb2 in $[\text{rnq}^{-}]$ cells overexpressing Sup35. Effects of wild type Lsb2 (8Q stretch), mutant Lsb2 QQ174, 175AA (4Q) or wild type Lsb1 (4Q) are compared.
Figure 2. Lsb2 Forms Punctate Aggregates, Partially Colocalized with Aggregated Sup35 and Induces the [RNQ⁺] prion

(A) Punctate structures were visualized in live cells by time-lapse microscopy after expressing Lsb proteins from the $P_{CUP1}$ promoter for 1.5 hrs. Distribution of Lsb2-GFP puncta, but not of Lsb1-GFP dots is polarized. See also Movies S1. Lsb2-GFP and Sup35NM-dsRED were co-expressed from $P_{CUP1}$ promoters for 14 hrs (B) and 93 hrs (C, D) in a $[psi^-]RNQ⁺$ strain, and analyzed by time-lapse confocal microscopy. Small aggregates of Sup35 fuse and grow into large aggregates in dynamic association with Lsb2-GFP (B), and increase their density with time (C). Z-stacks are from Movie S3 (B) and Movie S4 (C). See also Movie S2. (D) Lsb2-GFP puncta and Sup35NM-dsRED aggregates are separated and form independent structures. Z-stacks are from Movie S5. (E) Identification of the [RNQ⁺] prion, induced in the presence of excess Lsb2, by the “gel entry” assay. See also Figure S1E. Cell lysates were prepared from the original colony before induction, and from exceptional reinducible derivatives, retaining the ability to reinduce [PSI⁺] after its was cured with excess Hsp104 (see text). Equal amounts of lysates in 2% SDS were either incubated at room temperature or boiled for 5 min, and loaded onto SDS-PAGE. Lysates from a [RNQ⁺] strain are shown as a control. The prion form of Rnq1 is unable to enter the gel unless boiled, and is more abundant due to increased proteolytic stability, compared to the non-prion form.
Figure 3. Lsb levels and effects during stress
(A) Thermal stress induces Lsb2. See also Figure S2 and Movie S6. Pgk1 protein was used as a loading control. (B) Levels of Lsb2 induction by copper and heat shock. Protein levels were analysed at indicated time points using Lsb2 Ab. (C) [psi−] colonies are induced by heat shock. (D) [PSI+] destabilization and recovery during heat shock. (C,D) Yeast were grown to early exponential stage at 25°C, shifted to 39°C for specified period of time, then plated on YPD and incubated at 25°C. See also Figure S2C.
**Figure 4. Ubiquitination and degradation of Lsb proteins**

(A) HA-Lsb2 is expressed from *P_{CUP1} in the absence (no induction) or presence (induction) of additional CuSO₄. High MW conjugates of HA-Lsb2 are detected when protein is induced at high level along with Myc-Ub. (B) High MW conjugates of HALsb2, immunoprecipitated with anti-HA Ab, react to anti-Myc Ab, confirming that they contain Myc-Ub. (C) K80R substitution increases Lsb2 accumulation in the cell. HA-Lsb2 and its derivatives were detected with anti-HA Ab. Shorter (Exp1) and longer (Exp2) exposure times for the same blot are shown. (D) Substitution of a single lysine residue K80R in Lsb2 prevents accumulation of high MW conjugates, detected as on panel A. (E, F) HA-tagged Lsb1 and Lsb2 were expressed from the endogenous chromosomal promoter and proteins levels were compared in the wild type and *doa3-1 mutant strains, either without treatment (E) or at the indicated time points after the addition of cycloheximide (F).
Figure 5. E2 Enzymes of the Ubc1/4 Family and E3 Enzyme Rsp5 Are Involved in Lsb2 Ubiquitination

(A) ubc4Δ causes accumulation of HA-Lsb2 in the non-ubiquitinated form when protein is expressed from the P_CUP1 promoter at a low level, and reduces ubiquitination of Lsb2 when expressed at a high level with additional CuSO₄. See also Figure S3A. (B) Ubiquitination of HA-Lsb2 is decreased in the ubc1Δ strain. Proteins on panels A and B were analyzed as in Figure 4A. (C) Ubiquitination of HA-Lsb2 is abolished in the rsp5-1 mutant strain. Mutant and wild type cultures, grown in SD medium at 30°C, were shifted to medium with 100 μM CuSO₄ and incubated at 37°C (non-permissive conditions for rsp5-1) for 3 hrs. (D) Double P124A, P125A substitution in the potential Rsp5 binding site prevents HA-Lsb2 ubiquitination. (E) Defect in Lsb2 ubiquitination improves its [PSI⁺]-inducing ability. Wild type or mutant HA-LSB2 constructs were sequentially induced with P_GAL1-SUP35 in the LSB2 and lsb2Δ strains as described in Experimental Procedures, and [PSI⁺] induction was detected as in Figure 1A. See also Table S3.
Figure 6. The SH3 Domain Is Involved in Association of Lsb Proteins with the Actin Cytoskeleton and Ub, and in Prion Induction

(A) Yeast two hybrid assay. Protein fused to activation (AD) and DNA binding (BD) domains of Gal4 are shown. ‘Control’ refers to plasmids bearing the indicated domain not fused to another protein. Two-hybrid interaction is detected by activation of the $P_{GAL-AD}E2$ reporter construct, resulting in growth on –Ade (shown after 5 days of incubation). Results for the N-terminal PrD of Sup35, Sup35N (shown on Figure), and complete Sup35 (not shown) were identical. The W91S substitution in the SH3 domain abolishes interaction with Las17 and Ub, but not with Sup35 or Sup35N. (B) Purified HA-tagged Lsb2 or its SH3 domain were incubated with Ub, immobilized on beads, or beads alone as a control. Bound proteins were eluted and detected on an anti-HA immunoblot. (C) Co-localization of Lsb2-GFP and actin, stained by rhodamine phalloidin (Rd) in ethanol-fixed cells, was imaged by confocal microscopy. (D) Colocalization of Lsb protein with a marker protein for actin patches, Cap2 (as indicated by arrows). Also see Movie S7 and Figure S4. (E) Substitution of the conserved tryptophan (W) residue in the SH3 domain of Lsb1 or Lsb2 abolishes formation of punctate and aggregated structures. See also Figure S3B. (F) W91S mutant of Lsb2 does not promote [$PSI^+$] induction by excess Sup35. Experiment is performed as in Figure 1A. (G) Levels of the wild type and W91S mutant Lsb2 proteins are the same, as confirmed by Western blot with anti-HA Ab.
Figure 7. Model for the Stress Dependent Induction of Sup35 Prions at the Cytoskeletal Sites via Lsb2
See comments in the text.