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Graphical Abstract

Highlights

- Yeast protein Lsb2 forms a metastable prion inducing formation of other prions
- Thermal stress triggers formation of the metastable prion state of Lsb2
- Lsb2 has acquired prion activity in evolution due to a single amino acid change
- This change coincides with yeast adaptation to higher growth temperatures

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In Brief

Prions are transmissible protein aggregates. Chernova et al. show that a transient prion of yeast short-lived cytoskeletal protein Lsb2 is triggered by thermal stress and induces other prions. Evolutionary acquisition of prion-inducing activity by Lsb2 is traced to a single amino acid substitution, coinciding with yeast adaptation to higher temperatures.
Yeast Short-Lived Actin-Associated Protein Forms a Metastable Prion in Response to Thermal Stress

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SUMMARY

Self-perpetuating ordered protein aggregates (amyloids and prions) are associated with a variety of neurodegenerative disorders. Although environmental agents have been linked to certain amyloid diseases, the molecular basis of their action remains unclear. We have employed endogenous yeast prions as a model system to study environmental control of amyloid formation. A short-lived actin-associated yeast protein Lsb2 can trigger prion formation by other proteins in a mode regulated by the cytoskeleton and ubiquitin-dependent processes. Here, we show that such a heterologous prion induction is due to the ability of Lsb2 to form a transient prion state, generated in response to thermal stress. Evolutionary acquisition of prion-inducing activity by Lsb2 is traced to a single amino acid change, coinciding with the acquisition of thermostolerance in the Saccharomyces yeast lineage. This raises the intriguing possibility that the transient prion formation could aid in functioning of Lsb2 at higher temperatures.

INTRODUCTION

Many proteins can adopt an amyloid form, represented by ordered fibrous cross-β aggregates. In vivo, some amyloids are infectious or heritable through cytoplasmic inheritance during cell divisions. Thus, amyloids and prions are associated with a variety of human diseases. Some amyloid-associated diseases, such as Huntington’s disease (HD) and familial Creutzfeldt-Jakob disease (CJD), are caused by mutations (Shao et al., 2012). However, how prions arise in vivo and by which non-amyloid transmissible protein states in yeast (Liebman and Chernoff, 2012; Tuite and Serio, 2010; Wickner et al., 2016). Despite that some prions are clearly pathogenic to yeast cells (Wickner, 2011; Wickner et al., 2011, 2014), yeast prions are found in nature (Halfmann et al., 2012; Kelly et al., 2012; Nakayashiki et al., 2005; Resende et al., 2003).

Yeast prions are epigenetic determinants that alter multiple cellular processes and, as a result, significantly change the phenotype of the host. Thus a prion-based mechanism offers a potentially dynamic system for epigenetic regulation of phenotype in response to a changing environment. In yeast, prion formation or loss can be facilitated by environmental stresses including heat shock (Newnam et al., 2011; Tuite et al., 1981; Tyedmers et al., 2008). It has been hypothesized that transient prion-like states could be acquired in response to stress, where their presence could be protective and lost after these conditions have passed, although selfish or pathogenic self-perpetuating prions may remain as by-products of this process (Chernoff, 2007; Halfmann and Lindquist, 2010; Li and Kowal, 2012; Sugiyama and Tanaka, 2014). Prions whose biological effect depends on growth conditions include [MOT3⁺], which regulates expression of the genes affecting cell wall biogenesis and leads to filamentous growth and increased ethanol resistance ([Holmes et al., 2013]), and [MOD⁺], a prion isoform of an RNA modification enzyme that results in increased levels of intracellular ergosterol and resistance to inhibitors of ergosterol synthesis such as the common antifungal drugs fluconazole and ketoconazole ([Suzuki et al., 2012]). However, how prions arise in vivo and by which mechanisms stressful conditions influence prion formation remain largely unknown.

Yeast prion proteins usually contain glutamine (Q) and asparagine (N)-rich prion domains (PrDs) responsible for prion propagation ([Tuite, 2013]). Polymerization of a prion-forming protein,
resulting in formation of the initial prion “seed,” could be accelerated when the misfolded protein is present at a high local concentration. Indeed, de novo formation of yeast prions is induced by transient overproduction of a prion-forming protein or its PrD (Liebman and Chernoff, 2012). Such prion induction is significantly enhanced by the presence of other QN-rich prions, or by simultaneous overproduction of other yeast prions with QN-rich domains (Derkatch et al., 2001; Osherovich and Weissman, 2001). Some of these heterologous Q/N-rich inducers are known to form prions themselves, although such evidence is lacking for others (Alberti et al., 2009; Tuite, 2013). Functional interaction between PrDs of two yeast proteins, Pub1/Tia and Sup35 (Li et al., 2014), as well as promotion of polyglutamine aggregation by endogenous yeast prions (Gokhale et al., 2005; Merin et al., 2002), suggests that Q/N-rich proteins can be co-assembled in cellular locations that become prion nucleation sites. Interactions between various amyloidogenic proteins are also known to have been reported in mammalian systems, and some human amyloidoses (e.g., AD) involve formation of amyloids by more than one protein (Jucker and Walker, 2011; Walker and LeVine, 2012). Proposed (non-exclusive) models for heterologous prion induction include heterologous cross-nucleation or sequestration of folding cofactors (such as chaperones) promoting misfolding.

Recent data also indicate that prions may modulate degradation and posttranslational modifications of other prion-forming proteins (Yang et al., 2014). Deletions or alterations of some genes coding for chaperones and components of the ubiquitin proteasome system (UPS) involved in clearance of misfolded proteins were shown to promote de novo prion formation, specifically in the case of the Sup35 prion, [PSI+] (Chernova et al., 2014; Liebman and Chernoff, 2012; Massion and Reidy, 2015; Sporn and Hines, 2015). However, despite numerous studies (Allen et al., 2007; Chernova et al., 2003; Peng et al., 2003), no evidence for the regulation of Sup35 levels by ubiquitination has been reported. This suggests that UPS influences [PSI+] formation via ancillary proteins. Aggregating proteins capable of promoting nucleation of the heterologous prions are the most obvious mediators of the UPS interactions with a prion.

The Las17-binding protein 2 (Lsb2), also called Pin3 (from “prion induction”), is one of several QN-rich proteins that, when overproduced, are capable of promoting conversion of excess translation termination factor Sup35 into its prion form, [PSI+] (Chernova et al., 2011; Derkatch et al., 2001). Lsb2, together with its paralog Lsb1, also antagonize the loss of pre-existing Sup35 prion after heat shock (Ali et al., 2014; Chernova et al., 2011). Our previous finding that thermal stress dramatically increases cellular levels of Lsb2 (Chernova et al., 2011) points to the physiological relevance of these phenomena.

Here, we have directly tested and confirmed the hypothesis that induction of the Sup35 prion by Lsb2 is mediated by the formation of a metastable prion form of the Lsb2 protein itself. Moreover, our data show that formation of the Lsb2 prion is induced by thermal stress. This suggests that one possible role of metastable prions is to transiently modulate localization and aggregation of other proteins in the adaptation of yeast cells to a changing environment. Regulation of the transient Lsb2 by the actin cytoskeleton and UPS, ubiquitous throughout the eukaryotic kingdom, points to possible applicability of our results beyond yeast.

**RESULTS**

**Formation of a Heritable Metastable State [LSB+] by Lsb2 Protein**

It has been shown that yeast Sup35 protein, overexpressed in yeast cells lacking any pre-existing prion state [PSI+], frequently only if another protein with a QN-rich prion-like domain is co-overexpressed simultaneously (Derkatch et al., 2001). One such [PSI+]-inducing protein has been identified as an actin-associated short-lived protein Pin3/Lsb2 (Madiana et al., 1999) (Figure 1). We have demonstrated that even transient overproduction of Lsb2 at levels similar to those induced by heat shock promotes [PSI+] formation when Sup35 is either simultaneously or subsequently overexpressed (Chernova et al., 2011).

This phenomenon of “sequential” induction suggested that transiently overproduced Lsb2 generates “nuclei” that stay in the cell long after Lsb2 overexpression is turned off and trigger conversion of Sup35 into a prion [PSI+]. Now, we have proved this model by showing that indeed, Lsb2 forms [PSI+]-inducing aggregated nuclei that are heritable in a prion-like fashion. After transient overproduction of Lsb2 and return to normal levels of its expression, a fraction of cells (about 3%) gives rise to colonies in which excess Sup35 forms [PSI+] in the absence of continuous overproduction of Lsb2 (Figure 2A, left). No such colonies were detected in the control experiments without prior Lsb2 overproduction (Table 1). The [PSI+]-inducibility phenotype generated after Lsb2 overproduction was inherited through an indefinite number of mitotic divisions, albeit with low mitotic stability; only 2%–13% of cells from inducible colonies retained [PSI+] inducibility upon reploting. This phenotype required the continued presence of the LSB2 gene (Table S1): When it was initially generated in lsb2Δ cells with the LSB2 gene on a plasmid, the inducibility phenotype was lost in all of the colonies that have lost the LSB2 plasmid, whereas 5%–22% of colonies with plasmid retained inducibility (Table S1). However, when plasmid-containing inducible cells were mated to the haploid strain bearing normal chromosomal LSB2 gene, and the LSB2 plasmid was removed from resulting diploid cells, 7 out of 21 tested diploids formed [PSI+]-inducible colonies. This demonstrates that the chromosomal LSB2 gene with the endogenous promoter is sufficient to maintain the inducible state. Because [PSI+]-inducibility phenotype manifested itself as a heritable trait dependent on the Lsb2 protein, we have designated it here and further as [LSB+].

Total levels of Lsb2 protein were not increased in the [LSB+] cultures compared with isogenic non-inducible [lsb ] cultures (Figure 2B). However, by using semi-denaturing detergent agarose gel electrophoresis, SDD-AGE (Bagriantsev et al., 2006), we have shown that the [LSB+] cultures, but not [lsb ] cultures, contain a small but detectable fraction of the Lsb2 protein in the form of detergent-resistant polymers (Figure 2B). Such polymers were similar to, although less abundant than, those formed during Lsb2 overproduction (Figure 2C). Notably, [LSB+] cells retained another endogenous prion-forming protein,
Rnq1, in a soluble (non-prion) form (data not shown), confirming that the \( \text{PSI}^+ \) inducibility phenotype was not due to formation of the Rnq1 prion, \( \text{PIN}^+ \), that has previously been shown to promote \( \text{PSI}^+ \) induction (Derkatch et al., 2001).

To visualize Lsb2 aggregates in vivo, we mated \( \text{LSB}^+ \) and \( \text{lsb}^+/\text{C0} \) derivatives of the \( \text{lsb}^2 \text{D} \) strain, carrying the \( \text{LSB2} \) gene on a plasmid, to the haploid strain bearing both chromosomal \( \text{LSB2} \) gene and a plasmid, expressing mCherry-Lsb2 chimeric protein from the copper-inducible promoter. After removal of the \( \text{LSB2} \) plasmid from resulting diploids and induction of the expression of the mCherry-Lsb2 construct, both strains showed diffuse fluorescence throughout the cytoplasm and small puncta adjacent to the plasma membrane (Figure 2D, image on the right) in the majority of cells, as described previously (Chernova et al., 2011). However, only \( \text{LSB}^+ \) diploids formed bright single-clump mCherry-Lsb2 foci (Figure 2D, image on the left) in about 1% of the cells. This indicates that, at least in a fraction of cells, the \( \text{LSB}^+ \) prions can nucleate large, cytologically detectable foci.

Thus, our data show that the \( \text{PSI}^+ \)-inducible state, generated after transient Lsb2 overproduction and designated as \( \text{LSB}^+ \), depends on the Lsb2 protein, can be maintained at the endogenous levels of Lsb2 expression, and is associated with the heritable aggregated state of Lsb2, as would be expected of a yeast prion.

Modulation of the \( \text{LSB}^+ \) State by Ubiquitination, Cytoskeleton, and Chaperone Machinery

Lsb2 is a ubiquitinated protein; thus, we investigated the impact of ubiquitination on prion formation. Notably, mutant Lsb2 lacking the major ubiquitination sites, K80R and K41R, or missing the Rsp5 ubiquitin-ligase binding site, P124A and P125A, are not ubiquitinated (Chernova et al., 2011) and produce more abundant aggregates of Lsb2 compared with wild-type Lsb2 (Figure 2C). Although overproduction of ubiquitination-defective Lsb2 caused only slight increases in the frequency of induction of \( \text{LSB}^+ \) colonies compared with wild-type Lsb2 (Table 1), it should be noted that \( \text{LSB}^+ \) isolates produced by the ubiquitination-defective Lsb2 protein were characterized by increased mitotic stability compared with the \( \text{LSB}^+ \) isolates generated by wild-type Lsb2 protein. For example, \( \text{LSB}^+ \) was maintained in 53% ± 9% of progeny of the initially generated \( \text{LSB}^+ \) colonies in case of K80R, K41R Lsb2, compared with only 6% ± 4% in case of wild-type Lsb2. This difference was statistically significant (p < 0.001, Student’s t test). In contrast, the W91S mutation, which blocks Las17 binding and association of Lsb2 with the actin cytoskeleton (Chernova et al., 2011), also blocked the formation of detergent-resistant Lsb2 aggregates (Figure 2C) and \( \text{LSB}^+ \) (Table 1). These data agree with our previous observations indicating that the promotion of \( \text{PSI}^+ \) formation by overproduced Lsb2 is facilitated by a defect in Lsb2 ubiquitination and eliminated by disruption of actin association (Chernova et al., 2011). Our recent results explain the effects of these factors on \( \text{PSI}^+ \) induction by their effects on formation and propagation of the \( \text{LSB}^+ \) prion.

Most known amyloid-based yeast prions rely on the Hsp104 chaperone in order to propagate efficiently (Chernova et al., 2014; Liebman and Chernoff, 2012). In agreement with this generalization, we found that incubation of the \( \text{LSB}^+ \) culture for 20–40 generations on medium containing 5 mM GuHCl, a known inhibitor of Hsp104 (Ferreira et al., 2001; Jung et al., 2002; Ness et al., 2002), results in the loss of \( \text{LSB}^+ \). This effect was especially profound for the more stable \( \text{LSB}^+ \) isolate, formed by Lsb2 with K80R, K41R substitutions. In this case, all 64 tested colonies from the culture grown in the presence of GuHCl have lost \( \text{LSB}^+ \), whereas 26 (41%) out of 64 colonies from the culture grown for the same time in the absence of GuHCl retained it. This indicates that \( \text{LSB}^+ \), like most other

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**Figure 1. Alignment of Lsb1 and Lsb2 Proteins**

Corresponding residues in Lsb2 and Lsb1 essential for ubiquitination (K41 and K80, K79), binding to Las17 (W91, W90), and prion induction (N213, S239) are indicated. Blue bars represent “amyloid stretch” hexapeptides in Lsb2 and Lsb1.
known yeast prions, requires the Hsp104-based chaperone machinery for its inheritance.

**Meiotic Transmission of [**LSB**+]**

From 2% to 11% of diploid cells isolated after mating between the [**LSB**+] and [**lsb**] haploid cultures contained [**LSB**+]. However, most of the resulting [**LSB**+] diploid colonies exhibited 60%–80% prion retention in subsequent mitotic divisions, showing dramatically higher mitotic stability compared with [**LSB**+] haploids. To investigate the inheritance of [**LSB**+] in meiosis, we have analyzed three isogenic [**LSB**+] diploids that were homozygous for **lsb2**Δ, heterozygous for **mq1**Δ, carried **LSB2** on a single-copy (CEN) plasmid under the copper-inducible (PCUP1) promoter, contained an extra copy of **SUP35** on another CEN plasmid under the galactose-inducible (PGAL) promoter, and contained neither Rnq1 nor Sup35 prion. One of these diploids had wild-type hemagglutinin (HA)-tagged **Lsb2**, whereas two other diploids contained HA-tagged **Lsb2** with inactivated ubiquitination sites (K41R, K80R). All three diploids were maintained under conditions of low expression of **LSB2** (that is, in medium with only background levels of CuSO₄, 3 μM). Under these conditions, Lsb2 is produced at levels comparable with its normal cellular levels (Chernova et al., 2011).

These diploids were sporulated and dissected. The presence of [**LSB**+] was detected by [**PSI**+] formation after induction of **SUP35** overproduction on galactose medium in the spore clones.
Table 1. High Levels of Lsb2 Are Required for the Formation of [LSB⁺] Colonies

<table>
<thead>
<tr>
<th>Lsb2 High Levels</th>
<th>[LSB⁺] Colonies Tested</th>
<th>Lsb2 Low Levels</th>
<th>[LSB⁺] Colonies Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9 (2.7%)</td>
<td>WT</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>K80R</td>
<td>16 (2.7%)</td>
<td>K80R</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>K41R, K80R</td>
<td>10 (4.0%)</td>
<td>K41R, K80R</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>P124A, P125A</td>
<td>14 (3.1%)</td>
<td>P124A, P125A</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Q174A, Q175A</td>
<td>14 (1.8%)</td>
<td>Q174A, Q175A</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>W91S</td>
<td>0 (0.0%)</td>
<td>W91S</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>None</td>
<td>0 (0.0%)</td>
<td>None</td>
<td>0 (0.0%)</td>
</tr>
</tbody>
</table>

The culture [psi⁻ mq₇ lsb⁻] strain with the plasmids bearing the LSB⁺ gene wild-type or mutant under the copper-inducible and the SUP35 gene under the galactose-inducible promoter was induced for 24 hr with additional 100 μM copper († Lsb2, high levels) or without Lsb2, low levels are induced by residual copper present in the medium), plated onto the plasmid-selective medium and velveteen replica plated onto galactose medium († Sup35) followed by replica plating onto – Ade medium for detection of [PSI⁺] induction in the presumable [LSB⁺] colonies as shown in Figure 2A (left column).

that retained the SUP35 plasmid. As expected, the spore clones that have lost the LSB2 plasmid never exhibited [PSI⁺] induction, confirming that the LSB2 gene is required for [LSB⁺] inheritance in meiosis as well as in mitosis. Among spore clones retaining both LSB2 and SUP35 plasmids (from 10% to 50% of all spore clones depending on the diploid), heterozygous nuclear markers (mating type and mq1.J) segregated at ratios that were not statistically different from 50% (1:1), whereas [LSB⁺] showed irregular segregation characteristic of non-Mendelian inheritance. Overall, 14 (16.7%) out of 84 plasmid-containing spore clones retained [LSB⁺] after sporulation of the diploid with wild-type Lsb2, whereas 57 (22.4%) out of 255 plasmid-containing spore clones retained [LSB⁺] after sporulation of diploids with mutant Lsb2. The presence or absence of Rnq1 had no significant impact on [LSB⁺] inheritance, because [LSB⁺] colonies were detected with comparable frequencies among both RNQ1 (9 out of 44 for a diploid with wild-type Lsb2) and mq1.J (5 out of 40) spore clones. Haploid strains originated from spore clones with wild-type Lsb2 maintained [LSB⁺] in 15–20% of the mitotic progeny (60 colonies were checked for each of six spore clones tested), and mitotic stabilities were similar for the three mq1.J spore clones (15.6% on average) and the three RNQ1 spore clones (19.4%). The non-Mendelian mode of inheritance and high meiotic loss of [LSB⁺] are in agreement with what would be anticipated for a metastable prion.

Increase in [LSB⁺] Formation and Propagation by the Substitution of 8Q to 8N

Previously, it has been shown that the replacement of Q with N in poly-Q stretches of some yeast PrDs favors prion propagation (Halfmann et al., 2011). Lsb2 has a stretch of 8Q residues at the C terminus (Figure 3A). Replacement of this 8Q stretch of Lsb2 by 8N residues does not change the level of expression of Lsb2, but significantly increases the efficiency of [PSI⁺] induction in the presence of excess Lsb2 (Figures 3B and 3C) and decreases the apparent size of the Lsb2 detergent-resistant aggregates (Figure 3D), as is typical of more efficiently propagated prions (Liebman and Chernoff, 2012). Using a procedure described in Figure 2A, we demonstrated that transient overexpression of 8Qto8N Lsb2 produces more [LSB⁺]-containing colonies (94%; see Figure 3E), compared with wild-type protein (2.7%). Moreover, mitotic stability of the [LSB⁺] derivatives produced by the 8Qto8N Lsb2 protein is increased to 100% (Figure 3E).

Induction of the [LSB⁺] Prion by Heat Shock

We have shown previously that Lsb2 levels are increased by heat shock up to the levels comparable with overexpression (Chernova et al., 2011). This suggests that [LSB⁺] prion could be formed during heat shock. To capture heat-shock-induced [LSB⁺] prions, we have compared effects of heat shock on three yeast strains: (1) lsb2Δ strain, (2) wild-type strain, and (3) strain carrying the LSB2 gene with the 8Qto8N replacement on the chromosome under control of the endogenous Lsb2 promoter. The levels of the 8Qto8N Lsb2 derivative produced under normal conditions (Figure 4A) and during heat shock (Figure 4B) were similar to the levels of wild-type Lsb2. After yeast cultures were subjected to heat shock for 2 hr, individual colonies were tested for the [PSI⁺]-inducibility phenotype indicative of the [LSB⁺] prion. We identified [LSB⁺] colonies at frequencies of 0.1% (wild-type strain) and 0.6% (8Qto8N Lsb2 derivative); no [LSB⁺] colonies were identified in the lsb2Δ strain (Figures 4C and 4D). Notably, the majority of the [LSB⁺] isolates formed by both wild-type and 8Qto8N Lsb2 proteins were highly unstable. However, the 8Qto8N substitution increased the frequency of induction of the [LSB⁺] prion by heat shock. This effect, as well as lack of inducible colonies in the lsb2Δ strain, show that formation of inheritable [PSI⁺]-inducible colonies after heat shock strongly depends on the Lsb2 protein, thus confirming that the [LSB⁺] prion can be generated as a result of physiological changes in the Lsb2 levels, specifically in response to heat stress.

Sequence Requirements for Prion Induction by Lsb2

In spite of the 64% sequence identity between Lsb2 and its paralog, Lsb1, overproduction of Lsb1 does not promote conversion of excess Sup35 protein into a prion state (Chernova et al., 2011). Sequence homology between Lsb1 and Lsb2 is lowest in the middle regions of the proteins (Figure 1). In particular, Lsb2 contains a 60 aa region (124–183) including 28 Q or N residues, among them the above-mentioned 8Q stretch. In contrast, the corresponding region of Lsb1 consists of 74 aa, containing a total of 19 Q/N residues, and includes a stretch of only four Qs. To determine whether these differences impact prion nucleation, we constructed various chimeric Lsb1-Lsb2 proteins and compared their [PSI⁺]-nucleating abilities (indicative of the [LSB⁺] prion formation) after co-overproduction with Sup35 in a strain lacking pre-existing prions (Figure 5A). Expression levels of all chimeric proteins and wild-type Lsb1 and Lsb2 proteins were comparable with each other (Figure 5B). Although we have confirmed our previous observation (Chernova et al., 2011) that the 8Q stretch of Lsb2 contributes to prion induction...
We have surprisingly found that this stretch per se is neither essential nor sufficient for the induction. Data shown in Figure 5 demonstrate that the 8Q stretch is capable of promoting \( \text{PSI}^+ \) nucleation only in combination with the rest of the C-terminal region of Lsb2 (construct 11, Figure 5A), and not in combination with the C-terminal region of Lsb1 (construct 12, Figure 5A). Moreover, we have observed that the replacement of just the 32 aa C-terminal domain of Lsb1 with the corresponding sequence of Lsb2 (construct 14) enables \( \text{PSI}^+ \) induction (Figure 5).

Role of a Single Amino Acid Substitution in the Differential Prion-Inducing Properties of Lsb Proteins

Our data demonstrate that, although the N-terminal and SH3 domains of the Lsb proteins are interchangeable, only the C-terminal region of Lsb2 confers prion-inducing capability. Only 3 aa residues within the C terminus are different between Lsb1 and Lsb2 (see Figures 1 and 6A). To determine which of these substitutions modulate the prionogenic properties, we have performed site-directed mutagenesis at each of these positions. Not all mutations had significant effects on the level of protein expression (Figure 6B). The substitutions S230A or A231S within Lsb1 did not have any impact on prion formation. Surprisingly, however, changing residue 239 from serine (S) to asparagine (N) enabled Lsb1 protein to induce \( \text{PSI}^+ \) prion formation with efficiencies similar to Lsb2 (Figure 6A). The induction of \( \text{PSI}^+ \) by S239N Lsb1 also occurred in lsb2Δ cells, indicating that Lsb2 protein is not required for prion induction by mutant Lsb1 protein. Notably, the S239A substitution at the same position of Lsb1 did not confer the prion-inducing phenotype (data not shown), pointing out a significance of the N residue at this position. Moreover, S239N Lsb1 formed SDS-resistant aggregates, in contrast with wild-type or other mutant derivatives of Lsb1 (Figure 6C). The reciprocal N213S substitution in the Lsb2 protein completely abolished the ability of Lsb2 to induce \( \text{PSI}^+ \) prion formation and to form detergent-resistant aggregates (Figures 6A and 6B). Thus, the \( \text{PSI}^+ \)-inducing properties of mutants coincided with their abilities to form detergent-resistant protein aggregates at similar levels of protein expression (Figures 6C and 6D).

Notably, only the Saccharomyces sensu stricto clade contains two paralogs of Lsb proteins, whereas other Saccharomyces and related species possess only Lsb1. Moreover, even within the S. sensu stricto clade, only the Lsb2 proteins of S. cerevisiae and its sister species, S. paradoxus, contain N residue at the position 213, whereas Lsb2s of more distantly related species, S. mikatae and S. bayanus, bear S. In Lsb1, the S residue within the VNSIF motif is conserved in all 21 fungal species examined (data available at http://www.yeastgenome.org). This
suggests that the prion-inducing ability of Lsb2 is a relatively recent evolutionary acquisition.

**DISCUSSION**

**Lsb2 Protein Forms a Metastable Prion [LSB+]**

Previously we demonstrated that, in yeast, overproduction of a QN-rich heat shock inducible protein, Lsb2, promotes cytoskeleton-dependent prion formation by Sup35, another yeast protein with a QN-rich domain. We hypothesized that, because of its high local concentration at actin patches, Lsb2 itself can form a prion that then acts as a “seed” to nucleate prionization of Sup35. Here, we demonstrate that Lsb2 indeed can be converted to a heritable isoform upon physiological or artificial overproduction, presumably because of increased opportunities for the excess protein to misfold and aggregate. This isoform exhibits all the major features of a “classic” yeast prion: inheritance in mitotic divisions; dominance; non-Mendelian transmission through meiosis; and curing by GuHCl, an inhibitor of the yeast chaperone Hsp104 that is required for propagation of most known yeast prions. Notably, the presence of this heritable Lsb2 isoform strictly depends on the presence of the LSB2 gene, which is typical for prions, because mutations impairing ubiquitination and subsequent degradation of Lsb2 also increase the mitotic stability of [LSB+]. [LSB+] seems to be the only example of a metastable prion formed by a short-lived stress-inducible protein.

**Induction of the [LSB+] Prion by Stress**

Significantly, Lsb2 levels are greatly increased during thermal stress and then reduced because of its ubiquitination and proteasome-mediated degradation when yeast cells are recovering from the initial stress response. Notably, Lsb2 levels during heat shock are similar to those obtained in our overproduction experiments, both of which lead to the induction of [LSB+] prion. This raises a question whether formation of the prion form of Lsb2 can be induced by thermal stress. Indeed, we have phenotypically detected the [LSB+] prion in 0.1% of yeast cells after heat shock. The Lsb2 protein is essential for stress-induced [LSB+] formation; heat-shock-dependent induction of [LSB+] was completely eliminated by the deletion of the LSB2 gene and increased at about 6-fold by the substitution of the 8Q stretch with 8N, in agreement with data for other prions showing the increased N contents favors prion propagation.

The transient nature of both Lsb2 overproduction during stress and [LSB+] prion induced by this overproduction suggests that stress-inducible aggregation of Lsb2 may play a functional role in adapting to unfavorable conditions. Lsb2 prion formation and/or heterologous prion induction by Lsb2 require its concentration at specific locations, e.g., at the sites of actin patch assembly (see below). Our previous data (Chernova et al., 2011) demonstrated association of aggregated Lsb with actin patches (Chernova et al., 2011) and showed formation of aggregates by Lsb2-GFP expressed from endogenous chromosomal promoter during heat shock (Chernova et al., 2011). Our recent results...
confirm the lack of [LSB+] formation by the W91S mutant that is defective in binding to the actin cytoskeleton via Las17.

In fact, our data uncover a cellular role of Lsb2 during stress as a sensor of stress and inducer of other prions whose presence may afford a competitive advantage. This role of Lsb2 as a transient catalyst of heterologous prion formation is related to its ability to form a transient prion state that facilitates assembly of other aggregation-prone proteins at specific cytoskeleton-associated sites. This mechanism may have arisen as a protective tool intended to minimize the pathogenic effects of inducing misfolded proteins throughout the cell, and may also help prevent degradation of essential proteins under unfavorable conditions, as proposed previously (Chernoff, 2007). The reversibility of Lsb2 induction and metastable nature of the [LSB+] prion may help to reverse these processes once unfavorable conditions have passed or adaptation of yeast cells to these conditions has occurred. However, as a by-product, such a mechanism may generate self-perpetuating and potentially pathogenic amyloids transmitted in a prion-like fashion. Indeed, both require the association of Lsb2 protein with the actin cytoskeleton, which is modulated by the SH3 domain of Lsb2 (Figure 2C) (Chernova et al., 2011). This explains why the QN-rich domain of Lsb2 alone was not able to mediate formation of the prion state in the previous studies (Alberti et al., 2009).

A distant homolog of Lsb2 is a human G-receptor binding protein, Grb2, which anchors to a number of proteins involved in cell signaling and vesicular trafficking, including proteins regulating cytoskeletal dynamics (Tomas et al., 2014). Grb2 is known to interact with amyloidogenic proteins such as huntingtin (Baksi et al., 2014) and amyloid precursor protein (APP), a precursor of amyloid-β (Nizzari et al., 2007). Mutations in Grb2 are associated with neurodegenerative diseases and cancer. This indicates that our findings may be applicable beyond yeast. It is also possible that other auxiliary actin assembly proteins in other organisms are employed for the same purposes as yeast Lsb2.

Overall, our results demonstrate that formation of the prion form of Lsb2 occurs under physiological conditions without any artificial overproduction, requires association with the actin cytoskeleton.
cytoskeleton, and is induced in response to environmental stress. Because of its metastable nature, the [LSB+] prion appears to be lost quickly in the majority of cells upon return to normal conditions and resumption of mitosis. Alternatively, there may be loss of [LSB+] during adaptation to stressful conditions if Lsb2 protein levels are also decreased.

**Evolutionary Acquisition of Prion-Forming Properties by Lsb2**

Notably, Lsb2’s paralog, Lsb1, does not possess the ability to form amyloid and induce heterologous prions, despite the fact that it was previously shown to counteract the destabilization of [PSI+] prion by heat shock (Ali et al., 2014). Surprisingly, the difference in prion-inducing abilities of Lsb1 and Lsb2 proteins can be traced to a single amino acid change at position 239 (Lsb1)/213 (Lsb2) within the otherwise conserved C-terminal region. Lsb2 contains N at this position, whereas Lsb1 contains S. We demonstrated that the N213S substitution completely abolished the ability of overproduced Lsb2 to promote [PSI+] induction, which, as we showed above, depends on the formation of [LSB+] prion. Accordingly, the reciprocal S239N substitution enabled Lsb1 to induce [PSI+] formation. Remarkably, the residue 213/239 is located within a consensus of “amyloid stretch,” a hexapeptide sequence typically found in proteins forming amyloid aggregates in vitro (Pastor et al., 2007). Lsb2 possesses three while Lsb1 possesses two amyloid stretch hexapeptides, and S239N or N213S substitutions, respectively, create a third amyloid stretch in Lsb1 or knock out one of the amyloid stretches in Lsb2, whereas S239A or N213A substitutions (having no effect on prion formation) neither generate nor eliminate an amyloid stretch. Thus, the presence of the C-proximal amyloid stretch in Lsb correlates with the prion-inducing ability.

The Ross group previously demonstrated that the prion-forming activity could be artificially generated with as few as two mutations, suggesting that many non-prion Q/N-rich proteins may be just a small number of mutations from acquiring aggregation or prion activity (Paul et al., 2015). Our data, coupled with phylogenetic analysis of Lsb proteins, clearly demonstrates that such an acquisition has indeed happened in the process of yeast evolution. The gene duplication generating Lsb2 occurred only in the Saccharomyces sensu stricto clade, whereas other Saccharomyces-related species possess only Lsb1. Moreover, even among the S. sensu stricto yeast, only the Lsb2 proteins of S. cerevisiae and its most closely related sister species, S. paradoxus, bear an N residue at the position 213, whereas Lsb2s of more distantly related species bear an S residue at the respective position. This indicates that the prion-inducing activity of Lsb2 is a relatively recent evolutionary acquisition. Notably, S. cerevisiae and S. paradoxus represent a subgroup that is characterized by an increased optimal growth temperature and increased thermotolerance relative to other S. sensu stricto yeast (Gonçalves et al., 2011). The heat shock inducibility of Lsb2 production and the previously demonstrated...
involvement of this protein in the assembly of deposits of other aggregated proteins (Chernova et al., 2011) raises an intriguing possibility that the formation of the metastable Lsb2 prion could aid in the Lsb2 functioning at increased temperature and represents an adaptation of this protein to higher growth temperatures encountered by the S. cerevisiae /S. paradoxus branch.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions
The yeast strains of Saccharomyces cerevisiae and plasmids used in this study are described in the Supplemental Information (Tables S2 and S3).

Protein Analysis
Cells were lysed by boiling in SDS loading buffer or by vortexing with glass beads. Protein polymers were analyzed by semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) (Allen et al., 2005; Kryndushkin et al., 2003).

Plate Assay for [PSI+] Induction
The presence of [PSI+] was monitored by its ability to suppress the reporter ade1-14 (containing the premature stop codon, UGA), resulting in growth on media lacking adenine (–Ad) (Chernoff et al., 2002).

Heat Shock Experiments
Exponentially growing yeast cultures expressing wild-type or 8Qto8N mutant Lsb2 and containing a plasmid with SUP35 under Pgal1 were grown in YPD medium at 25°C and shifted to 39°C (Newnam et al., 2011). Cell aliquots were collected at the specified time points and plated onto synthetic dextrose (SD) selective for Lsb2 and containing a plasmid with PGAL1-SUP35 under C14/C0 medium at 25°C and shifted to 39°C (Chernoff et al., 2007). Hsp70 chaperones are modulators of prion life cycle: novel effects of Ssa and Ssb on the Saccharomyces cerevisiae prion [PSI+]. Genetics 169, 1227–1242.

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Data Analysis
All experiments were performed at least in triplicate. The results are presented as mean ± SD. Differences were considered statistically significant if p < 0.05 according to Student’s t test (McDonald, 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.12.082.

AUTHOR CONTRIBUTIONS


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