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Divergence in Prion Inducing Ability of Paralogous Actin Asssociated Proteins

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# Divergence in Prion Inducing Ability of Paralogous Actin Associated Proteins

By

Moiez Ali

Advisor: Keith D. Wilkinson, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Master of Science

Biology

2012

Abstract

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By Moiez Ali

Prions are infectious, amyloid-like protein aggregates that transmit neurodegenerative diseases in mammals and heritable traits in yeast. It is important to study the formation of prions to better understand the progression of prion diseases and related neural inclusive diseases including Alzheimer, Parkinson, and Huntington. Although the precise mechanism of initial prion formation remains unclear, *de novo* formation of a yeast prion is induced by transient overproduction of the prion-forming protein and is efficient only if other Q/N-rich protein aggregates are present in the same cell. Recently, it has been shown that overexpression of Q/N-rich protein Lsb2 (Las seventeen binding protein 2) promotes conversion of translation termination factor Sup35 into its prion form, [*PSI*$^{+}$]. In contrast, Lsb1, a paralogous protein which shares 64% amino acid identity with Lsb2 does not promote [*PSI*$^{+}$] formation. Here, we show that structural and sequence differences between the Lsb proteins may be responsible for facilitating Lsb2 prion inducing ability. However, we provide evidence that suggests that Lsb1 protein may regulate the prion inducing ability of Lsb2 through a direct association. In addition, we demonstrate that the protein levels and stability of the Lsb proteins depends on the presence of Las17, an actin polymerization factor, and are regulated by ubiquitination. Loss of either *LSB1* or *LSB2* results in destabilization of a weak [*PSI*$^{+}$] variant under mild short-term heat shock. Together, this data provides evidence to show that the Lsb proteins may be involved in the formation and segregation of protein aggregates, indicating a possible biological significance of the Lsb proteins. Also, our findings elucidate the role of actin cytoskeleton machinery and ubiquitin proteolysis in regulating prion induction.

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First and foremost, I would like to acknowledge and give recognition to both Keith D. Wilkinson and Tatiana A. Chernova for their constant guidance throughout my studies at Emory University and for their intellectual contributions throughout this work.

In addition, I would like to convey my gratitude for the assistance of the following individuals: Luming Yin for protein purification of Lsb1, Oskar Laur (Emory CCCF) for plasmid construction, and Nick Seyfried (Emory Proteomics Core) for Mass Spectrometry analysis of Lsb1 protein.

# Chapter 1 General Introduction

## *Prions*

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) The propagation and transmission of prions result from repeated rounds of templating; a misfolded conformation of a protein converts additional molecules of the same protein from a normally folded state into an alternate conformation that aggregates within the cell. These aggregates, when inherited, are able to convert newly synthesized molecules of the normal protein into the infectious, misfolded form. Thus, prions represent a form of heritable variation not based on alterations of the genetic material, but instead, on an epigenetic switch determined by changes in protein structure (Uptain and Lindquist, 2002).

In humans, prions are associated with the development of several neurological disease states, including various transmissible spongiform encephalopathies (TSE) such as Creutzfeldt-Jakob disease, fatal familial insomnia and kuru (Chiti and Dobson, 2006). Similarly, there are several, well-characterized prions in *Saccharomyces cerevisiae*. These include [*URE3*], [$RNQ^{+}$], and the best-studied yeast prion, [$PSI^{+}$], an aggregate of the translation termination factor Sup35 (Liebman and Derkatch, 1999).

Sup35 protein is a translation termination factor, also known as eRF3, which in its native conformation functions with eRF1 at the ribosome to promote efficient termination at stop codons and release of polypeptide chains (Liebman and Derkatch, 1999). Upon conversion to its prion form, [$PSI^{+}$], Sup35 can no longer perform its normal function.

Instead, in [*PSI*$^{+}$] cells, there remains significantly less non-aggregated Sup35 available to function in translation, resulting in insufficient termination and the phenotype of nonsense suppression. Recent studies have attempted to define the biological significance of [*PSI*$^{+}$] prions. The results suggest that prions may lead to the acquisition of diverse and advantageous phenotypes that may be beneficial under selective pressure (Halfmann et al., 2012).

In mammals and yeast, prion assembly involves the formation of ordered cross-beta sheet structures, called amyloids, which, through several rounds of sequence-dependent polymerization, form insoluble aggregates within the cell (Sipe and Cohen, 2000). Of interest is the close resemblance of these prion aggregates to the amyloid structures that are observed during development and progression of neural inclusive disorders, including Alzheimer's, Parkinson's and Huntington (Jucker and Walker, 2011). In many ways, the prion replication process in yeast recapitulates elements of the amyloid formation process observed in numerous human proteopathies where deposition of ordered amyloid fibrous protein aggregates can result in disruption of normal cellular function.

## *Life cycle of a prion*

There are three events that characterize the lifecycle of prions in yeast: formation of prions, propagation of prion aggregates, and curing (or loss) of prions.

## Formation

Although spontaneous appearance of prions in laboratory strains of yeast occurs at a low frequency, *de novo* appearance of prions can be aided by the overexpression of

the entire prion proteins or distinct regions which are essential for propagation, termed prion domains (PrDs) (Chernoff, 2007; Derkatch et al., 1996). Mutations of the Sup35 protein within the N-terminal prion domain (PrD) or, alternatively, deletion of its PrD can promote the loss of [*PSI*$^{+}$]. In the case of Sup35, the prion domain contains an abundance of glutamine (Q) and asparagine (N) residues that resembles the poly-Q rich region of proteins such as huntingtin, which is involved in Huntington's disease (Inge-Vechtomov et al., 2007). Interestingly, the process of [*PSI*$^{+}$] prion formation via Sup35 overexpression is efficient only in the presence of other Q/N-rich protein aggregates such as [*RNQ*$^{+}$], the prion form of the Rnq1 protein (Derkatch et al., 1997). This effect is thought to be a result of preexisting Q/N-rich aggregates serving as primary nucleation centers for aggregation of the Sup35 Q/N-rich protein. Once formed, Sup35 aggregates can template the amyloid conformation through the prion domains of additional Sup35 molecules, forming insoluble fibers. Remarkably, assembly of amyloid fibers like those generated by Sup35 resembles the assembly of cytoskeletal structures such as actin filaments. Actin filaments (or microfilaments) form part of the cytoskeleton (CSK) by polymerization of actin subunits. CSK is a cellular scaffold that serves numerous key functions in eukaryotic cells, including facilitating intracellular transport, cell division, and cell polarity (Toret and Drubin, 2006).

In spite of this general understanding, little is known about the precise mechanism by which initial prions arise from the normal conformation of prion proteins *in vivo*. Studies in yeast have shown that the prion domain of Sup35 interacts with several components of the cytoskeletal machinery, and that depletion of vesicle assembly machinery or mutational alteration of actin affects aggregation of Sup35 (Ganusova et al.,

2006). Current models propose that Sup35 aggregates assemble initially in cortical actin patches, which are major sites of endocytic vesicle assembly, and are then moved inside of the cell along with components of the endocytic machinery (Ganusova et al., 2006). Therefore, it is likely that the actin cytoskeleton plays a central role in *de novo* prion formation by virtue of its ability to promote aggregation and transport of misfolded proteins.

## Prion induction by heterologous proteins

To identify factors that affect the rate of prion formation, Derkatch et al. conducted a screen of proteins containing prion-like domains that could promote induction of [*PSI*$^{+}$] prion (Derkatch et al., 2001). Among the dozen or so identified proteins that could induce the [*PSI*$^{+}$] prion was Pin3 ([*PSI*$^{+}$] inducing), also known as Lsb2 (Las seventeen binding protein 2). In contrast, a very close paralog, Lsb1, was not identified as one of these prion-inducing proteins. However, one limitation of this screen was that it was unable to show *de novo* appearance of [*PSI*$^{+}$] by Lsb2 protein. Additionally, it remains uncertain whether overexpression of Lsb2 protein can induce formation of stable prions, although, based on preliminary alignments of the Lsb2 protein structure, it remains possible that *LSB2* could be a prion gene. Therefore, one focus of this study is to analyze the prion inducing ability of Lsb2 and its structural homologue Lsb1.

A role for Lsb2 was first suggested by a large-scale yeast two-hybrid analysis used to identify protein binding partners of the Las17 protein, where six proteins of unknown function were discovered, including both Lsb1 and Lsb2 protein (LSB=Las

seventeen binding) (Madania et al., 1999). Apart from their association with Las17, the cellular function of the Lsb1 and Lsb2 proteins has not yet been revealed. However, these Las seventeen binding proteins exhibit 64% amino acid identity. Here, we will examine other key characteristics of these proteins that may play a fundamental role in their functional presence as well as regulation in yeast.

Lsb1 and Lsb2 proteins were also identified as ubiquitinated proteins such in a large scale analysis in yeast (Peng et al., 2003). Ubiquitination is a post-translational modification of proteins and generally signals abnormal or damaged proteins for degradation via the ubiquitin-proteasome system (UPS) (Hershko and Ciechanover, 1998). Through the sequential action of several enzymes, including ubiquitin conjugating (Ubc) enzymes and ubiquitin ligases, an ubiquitin molecule is transferred to its target protein (Graph 1). Ubiquitin and the target protein are connected by an isopeptide linkage involving the lysine residue of the protein substrate and the C-terminal glycine residue of ubiquitin. Failure of UPS due to mutations of the different components can lead to aggregation of misfolded proteins, which in turn can inhibit the function of remaining UPS components (Bence et al., 2001). In particular, it has been shown that alterations of various components of the UPS affect [$PSI^{+}$] prion formation and propagation (Allen et al., 2007; Chernova et al., 2003). Although ubiquitin is found enriched within the intracellular deposits of prion aggregates, ubiquitination of yeast prion proteins has not been detected. However, it has been proposed that UPS affects prion maintenance through the ubiquitination of auxiliary proteins. Furthermore, alterations of UPS could result in the accumulation of large, "aggresome"-like aggregates that can promote *de novo* prion formation (Allen et al., 2007; Tyedmers et al., 2010). Here, we investigate if

Lsb1 and Lsb2 proteins are regulated via ubiquitination. We will also determine the effect of ubiquitination on prion induction of the Lsb proteins.

## Loss or "curing" of a prion

If the daughter cell fails to inherit prion seeds during cell division, the prion will gradually be lost from the population. Classical yeast prions fail to form when the heat shock chaperone Hsp104 is overexpressed, perhaps because aggregates can never reach a size to act as seeds for prion propagation. Conversely, prions are rapidly lost from the population when Hsp104 is absent or inhibited by low levels of guanidinium hydrochloride (Chernoff et al., 2002).

Previous studies have reported that continuous growth at 37°C does not affect [*PSI*$^{+}$] phenotype, whereas severe heat shock at 50-55°C results in slight loss of [*PSI*$^{+}$] (Cox et al., 1988). However, effects of severe heat shock are difficult to study due to high cell death of the yeast population. This difficulty has also led to contradictory data concerning the effect of [*PSI*$^{+}$] on the cytotoxic consequences of heat shock (Ferreira et al., 2001; True and Lindquist, 2000). Nevertheless, recent data has shown that short-term mild heat shock results in gross destabilization of [*PSI*$^{+}$] and that stability is recovered after longer incubation at elevated temperatures (Newnam et al., 2011).

Mild heat shock conditions impair prion segregation, resulting in a bias towards prion retention in the mother cell. This imbalance has been proposed to be the result of an altered balance between Hsp104 and other stress-inducible heat-shock proteins (Hsps). Of particular interest is the role Hsp104 protein plays in the retention of protein aggregates, where the actin cytoskeleton has been reported to serve as a scaffold that

prevents inheritance of Hsp104-containing aggregates (Liu et al., 2010). Here, Hsp104 is thought to localize misfolded proteins to the mother cells and reduce the inheritance of proteotoxic aggregates to daughter cells (Erjavec et al., 2007). Therefore, since the Lsb proteins associate with Las17, which plays a key role in both actin patch assembly and the polarized distribution of actin, it is possible that Lsb2 and Lsb1 may function within the processes that govern spatial quality control (SQC) and segregation of damaged/aggregated proteins.

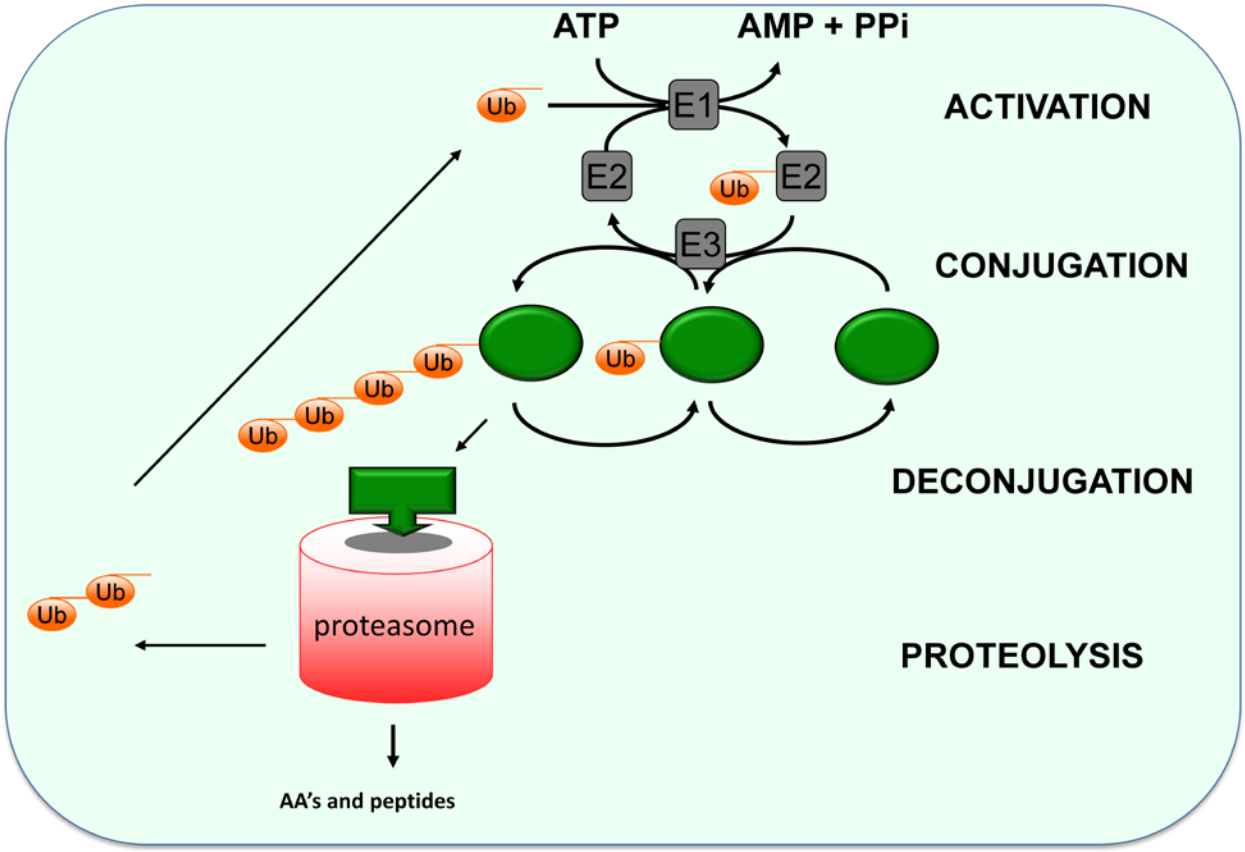
## *Involvement of the Actin cytoskeleton*

Considerable evidence, including that described here, point to a central role of the actin cytoskeleton in the life cycle of a prion. Actin patches are the sites of actin polymerization and depolymerization at the cellular membrane. Here, the Arp2/3 complex binds to existing filaments and initiates growth of new filaments at a 70-degree angle. Specifically, the Arp 2/3 complex functions in the creation of actin nucleation centers, necessary for the initiation of actin filament formation (Suetsugu et al., 2002). This nucleation activity is facilitated by members of the Wiskott-Aldrich syndrome (WAS) family of proteins that include mammalian Wiskott-Aldrich Syndrome protein (WASP), neuronal WASP (N-WASP), and the *S. cerevisiae* WASP homolog, Bee1/Las17 protein (Millard et al., 2004). Mutations of the WASP encoding gene result in the immune-deficiency Wiskott-Aldrich syndrome in humans. In yeast, deletion of *LAS17* results in the non-polarized distribution of cortical actin patches, as well as defects in budding and cytokinesis (Li, 1997; Rajmohan et al., 2009). It has been proposed that Las17 most likely functions by integrating multiple regulatory cascades destined for the Arp2/3 complex and actin cytoskeleton (Madania et al., 1999). Given its role in

regulating actin dynamics, Las17 and proteins like Lsb1 and Lsb2 that associate with Las17 could serve a role in the generation of Sup35 aggregates, as well as the partitioning of prions between mother and daughter cells (Verges et al., 2011).

## *Scope of this thesis*

Here, we will investigate the prion inducing ability of the Lsb proteins in the presence of Sup35. We will also study the effect of mild heat shock (39°C) on the maintenance of [*PSI*$^{+}$] prion in the presence and absence of the Lsb proteins. Furthermore, we will analyze specific characteristics of both Lsb2 and Lsb1, including ubiquitination and cellular associations, which may regulate their cellular function in yeast. With this data, we will propose what biological significance the Lsb proteins have in both the formation and segregation of protein aggregates. This study attempts to further our understanding of the processes of protein aggregation and prion propagation in yeast, which can serve as a model for the development and progression of protein misfolding disorders in humans.




Graph 1. Process of Ubiquitination and UPS-dependent degradation of proteins

# Chapter 2 Promotion of *de novo* induction of [*PSI*$^+$] by Lsb2 and Lsb1

## Introduction

Lsb1 and Lsb2 proteins were initially discovered in a large scale screen (yeast two-hybrid analysis) used to identify protein binding partners of the Las17 protein. Las17 is involved within actin patch assembly and actin polymerization via the Arp2/3 complex in yeast (Madania et al., 1999). Apart from their association with Las17, little is known as to the cellular function of Lsb1 and Lsb2. However, Lsb2 has been shown to be able to induce [*PSI*$^+$] prion upon overexpression of Sup35. Lsb1 and Lsb2 proteins exhibit 64% amino acid identity. Here, we will examine the role of the Lsb proteins in prion induction using Sup35 as the indicator of prionogenesis and discuss key characteristics of these proteins which may contribute to this function.

## Results

### Lsb2, but not Lsb1, induces the formation of [*PSI*$^+$] via Sup35

*LSB2/PIN3* was first identified in a screen for proteins that can induce [*PSI*$^+$] prion formation by Sup35 when both Sup35 and the candidate protein were overexpressed (Derkatch et al., 2001). Recently it was also identified in a bioinformatics screen for prionogenic proteins (Alberti et al., 2009). Lsb2 and its homologue protein Lsb1 share 64% identity and similar structural organization (Madania et al., 1999). The strongest homology between Lsb2 and Lsb1 is at the N-terminal containing SH3 domain and the last 30 residues at the C-terminal (Figure 1A). The central parts of these homologs have little similarity. Lsb2 contains a central 60 amino acid prion-like domain (PrD) with a total of 28 Q/N residues, including eight adjacent Q residues. The corresponding region of Lsb1 consists of 74 residues with total of 19 Q/N residues, but

only four adjacent Q residues.

To further our understanding of properties required for prion induction, we analyzed and compared the prion inducing ability of Lsb2 and Lsb1. We cloned a chromosomal copy of *LSB2* and *LSB1* into the pmCUP plasmid under control of a copper inducible promoter $P_{CUP1}$. Using the nonsense suppression assay, we have confirmed the [*PSI*$^+$]-promoting capability of Lsb2 in cells whether we co-overexpressed Lsb2 and Sup35 simultaneously or sequential transient overexpression of Lsb2 and then *SUP35* (Figure 1B). In both cases, cells were transferred to glucose medium lacking adenine (-Ade), where both constructs are turned off, and [*PSI*$^+$] induction was detected by growth. The Ade$^+$ colonies were confirmed to contain the [*PSI*$^+$] prion by demonstrating that the Ade$^+$ phenotype is curable by growth in the presence of guanidine chloride (GuHCl) and by overproduction of the chaperone Hsp104, agents known to eliminate the [*PSI*$^+$] prion (Chernoff et al., 2002).

We used the same approach to test the ability of overproduction of Lsb1 to induce [*PSI*$^+$] prion upon over-expression of Sup35. Despite the high homology to Lsb2, Lsb1 does not induce [*PSI*$^+$] prion by over-expression of Sup35 (Figure 1B). The major differences between Lsb2 and Lsb1 proteins are found in the sequence of the central part of these homologs, where Lsb2 contains a higher proportion of Q/N residues and possesses a stretch of eight adjacent Q residues, in contrast to only four at the comparable position of Lsb1. To ask if the length of the poly-Q repeat was relevant, we used site-directed mutagenesis to shorten the poly-Q repeat in Lsb2 from eight to four residues by substitution of the third and fourth Q residue with alanine residues, generating pmCUP-*LSB2* Q174A,Q175A. We tested the prion inducing ability of this mutant as described

above and found that this alteration reduces efficiency of prion induction but does not completely abolish it (Figure 1B). Furthermore, we used mutagenesis to generate pmCUP-*LSB2* Q172A,Q173A,Q174A,Q175A, to see if we could further reduce the efficiency of prion induction. However, this mutant behaved similarly to the 4 poly-Q repeat mutant (Figure 1B). This result indicates that, in addition to the size of poly-Q stretch, there are other sequence features of Lsb2 sequence that are involved in prion induction.

**Discussion**

The focus of future experiments will be to analyze the various domains and structure of the Lsb proteins to more fully understand the characteristics of Lsb2 that enable prion induction, and to understand how differences within the Lsb1 protein prevent its ability to induce [*PSI*$^{+}$] prion. By investigating the differences between these two proteins, we can begin to address the question of how Lsb2 induces prions and determine if Lsb1 plays any role in the prion formation process mediated by Lsb2. Although Lsb1 is not currently directly implicated in prion formation, given the close conservation of characteristics and structural properties to Lsb2, it remains possible that Lsb1 has evolved a regulatory role in the prion induction process, acting to modulate the activity of Lsb2 in the cell.

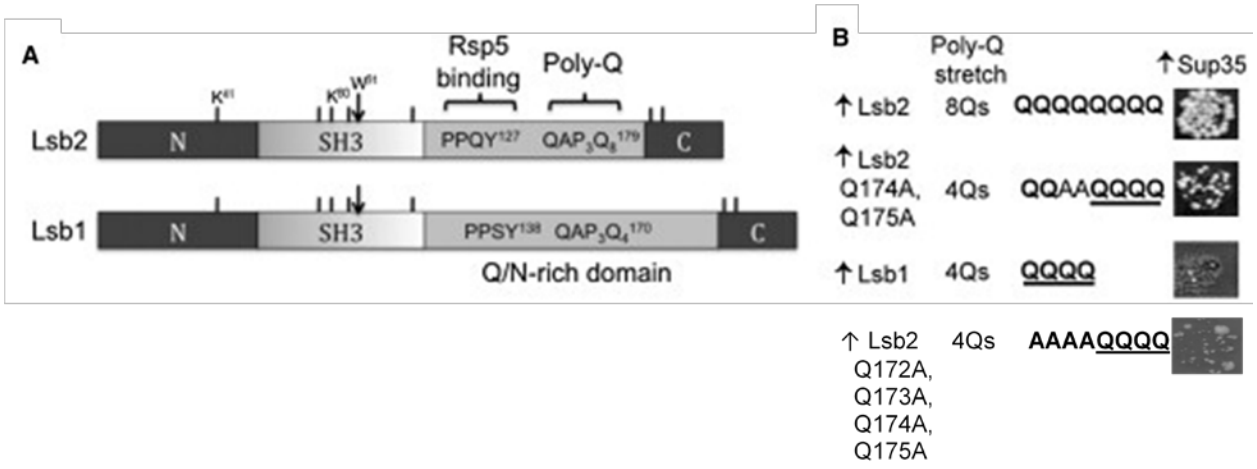



Figure 1. Homologous Proteins Lsb2 and Lsb1 Differ in Prion-Inducing Ability

(A) 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 correspond to amino acid positions and the number of repeated residues in a stretch, respectively. (B) Poly-Q stretch length influences the [*PSI*$^{+}$] promoting capabilities of Lsb2 in cells overexpressing Sup35. Effects of wild-type Lsb2 (8Q stretch), mutant Lsb2 QQ174, 175AA (4Q), mutant Lsb2 QQQQ172, 173, 174, 175AAAA and wild-type Lsb1 (4Q) are compared.

# Chapter 3 The Lsb proteins are both ubiquitinated

## Introduction

Ubiquitination of proteins is a reversible, post-translational modification whereby the ubiquitin (Ub) peptide is covalently linked to a lysine residue in the substrate protein (Hershko and Ciechanover, 1998). Both monoubiquitination and formation of polyubiquitin chains on proteins have been implicated in various cellular processes, including directing localization of cellular substrates, antigen presentation, and, most well known, targeting proteins for degradation via the proteasome (Pickart, 2001). Of particular interest is the role of ubiquitination in signaling misfolded proteins for degradation, as this could play a modulatory role in the retention and/or destruction of prion aggregates upon stress-induced protein misfolding response or impairment of UPS-dependent degradation of misfolded proteins (Goldberg, 2003).

## Results

### Lsb1 is ubiquitinated in the cell

Large-scale mass spectrometry analysis has revealed Lsb1 and Lsb2 are ubiquitinated on residues K41 and K79 in Lsb1 and K80 in Lsb2 (Peng et al., 2003). Previously, we have confirmed that Lsb2 undergoes ubiquitination primarily at one amino acide residue, K80. To analyze ubiquitination of Lsb1, we induce expression of HA-tagged Lsb1 at high levels under the P*CUP1* promoter similar to Lsb2. We detect the appearance of similar high-MW bands, resembling a ladder of ubiquitinated protein (Figure 2A). The K79R mutation of Lsb1 abolishes the appearance of high-MW conjugates of Lsb1, while expression of the K41R mutation shows the same high-MW protein ladder observed when wild type Lsb1 is expressed. Therefore Lsb1 is similar to

Lsb2 in that it is ubiquitinated mainly on one lysine residue, K79.

**Lsb1 is ubiquitinated by Rsp5 E3 ligase**

It was previously reported within a global proteomic screen that Lsb1 is a substrate for the E3-Ub ligase Rsp5, and that ubiquitination is dependent upon the presence of a "PY motif" within the substrate (Gupta et al., 2007). To confirm that Rsp5 is in fact the E3 ligase responsible for ubiquitination of Lsb1 protein, we expressed HA-tagged Lsb1 in wild type *RSP5* and mutant *rsp5-1* strains to observe if alterations of Rsp5 function resulted in the abolishment of high-MW bands of Lsb1. Indeed, ubiquitinated forms of Lsb1 were no longer present in the *rsp5-1* mutant strain (Figure2B). Furthermore, Lsb1 protein appears to accumulate when Rsp5 function is abolished. We next sought to determine if mutation of key proline residues within the putative Rsp5 recognition motif (the "PY motif" of Lsb1) would prevent ubiquitination and abolish the presence of high-MW conjugates of this protein. By expressing Lsb1 bearing a mutation within the putative Rsp5 binding site (alanines were substituted for the adjacent proline residues, P135A, P136A in Lsb1), Lsb1 ubiquitination was similarly abolished (Figure 2C). Previously, we have demonstrated that Lsb2 is ubiquitinated by Rsp5 (Chernova et al., 2011). Here, our data show that Lsb1 ubiquitination is also dependent on Rsp5.

**Lsb2 and Lsb1 protein half-life is influenced by alterations in the proteasome**

One of the possible roles of the ubiquitination of the Lsb proteins could be to target Lsb2 and Lsb1 for degradation via the ubiquitin proteasome system (UPS). By analyzing expression of HA-tagged Lsb1 and Lsb2 from the endogenous chromosomal promoter within wild type and *doa3-1* proteasomal mutant strain, we observed a significant accumulation of both Lsb1 and Lsb2 in the proteasome deficient strain (Figure

2D). We also observed that the half-life of Lsb2 was increased from 10-15 minutes to over 40 minutes in the doa3-1 mutant strain, and a similar trend was observed for Lsb1 (Figure 2E).

**Discussion**

Overall, these data show that both Lsb1 and Lsb2 are ubiquitinated within the cell, and that the process of ubiquitination involves the Rsp5 E3 ligase. Further, both proteins are stabilized by preventing ubiquitination and by interfering with proteasomal function. These observations implicate the regulation of the Lsb proteins via an ubiquitin-mediated degradation. This postranslational modification could play a key role in regulating protein stability and half-life of Lsb1 and Lsb2.

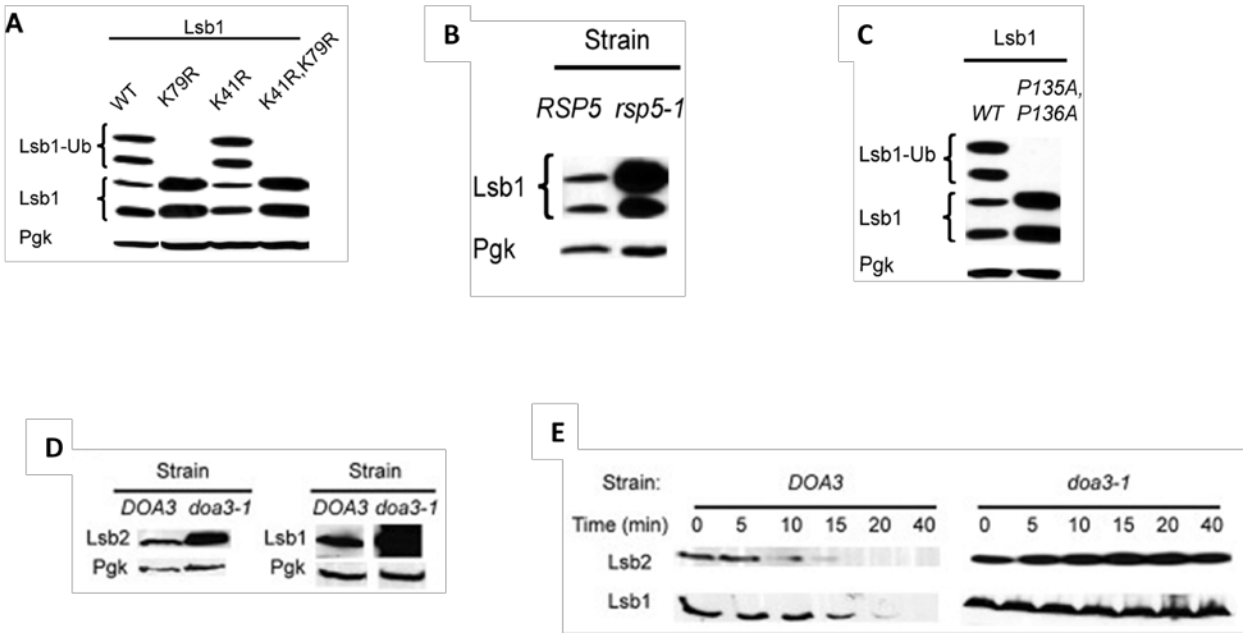



Figure 2. Ubiquitination and Degradation of the Lsb Proteins

(A) High molecular weight (MW) conjugates of HA-Lsb1 are detected with anti-HA Ab when protein is induced at high levels from $P_{CUP1}$ promoter. Substitution of a single lysine residue K79R in Lsb1 prevents accumulation of high-MW conjugates. Pgk is used as a loading control. (B) Ubiquitination of HA-Lsb1 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 $CuSO_4$ and incubated at 37°C (nonpermissive conditions for *rsp5-1*) for 3 hr. (C) Double P135A, P136A substitution in the potential Rsp5 binding site prevents HA-Lsb1 ubiquitination. (D and E) HA-tagged Lsb1 and Lsb2 were expressed from the endogenous chromosomal promoter, and protein levels were compared in the wild-type and *doa3-1* mutant strains, either without treatment (D) or at the indicated time points after the addition of cycloheximide (E).

# Chapter 4 Analysis of protein binding partners of Lsb2 and Lsb1

## Introduction

In order to understand the mechanism of prion induction via Lsb2 and/or any physiological role of Lsb1 and Lsb2 in yeast, it is imperative to understand associations of Lsb1 and Lsb2 with cellular compartments and cellular constituents. Here, we use several detection methods, including both *in vivo* and *in vitro* experiments, to detect new and confirm known protein interactions of both Lsb1 and Lsb2 proteins.

## Results

### Lsb2 and Lsb1 bind Ub, Las17 and Sup35

We utilized the yeast two-hybrid system to observe protein interactions in yeast. For this analysis, we introduced wild type Lsb2 and Lsb1 into the Gal4-DNA Activating Domain (pGAD) vector, with Ub, Las17, Sup35 and Sup35N (N-termimal PrD domain only) being fused to the Gal4-Binding Domain (pGBDU vector) (James et al., 1996). Here, we confirm that both Lsb proteins bind to Ub and Las17, and that both Lsb proteins bind Sup35 and Sup35N (Figure 3A). Next, we sought to determine the site of the interaction between the Lsb proteins and the identified binding partners. Mutation of the conservative hydrophobic W91 residue to the hydrophilic serine (S) amino acid located within the SH3 domain abolished the interaction of Lsb2 with both Ub and Las17 but did not affect interaction with Sup35N. In contrast, the conservative W90S mutation of Lsb1 protein abolished binding to Las17, while only slightly affecting interaction with Ub, and having no affect on its interaction with Sup35N. This suggests that there may be other domains or residues which contribute to Ub binding to Lsb1. Our data indicate that the Lsb proteins bind Las17 and Ub via their N-terminal SH3 domains, while their

interaction with Sup35N involves a different domain (possibly, the Q/N-rich, PrD-like region).

**Lsb2 and Lsb1 interact *in vivo***

Due to their ability to associate with many similar cellular proteins, as well as the list of conserved characteristics of both Lsb proteins, we sought to determine if Lsb1 and Lsb2 interact with each other. Once again, using the yeast two hybrid assay, we show that Lsb1 and Lsb2 exhibit an interaction in yeast. Furthermore, this interaction is not abolished by mutation of the W residue to S located in the N-terminal SH3 domain of both Lsb1 and Lsb2 (Figure 3B). This clearly suggests that other regions of both Lsb proteins mediate their interaction.

**Lsb2 and Lsb1 interact *in vitro***

We next sought to determine if we could observe the interaction among the Lsb proteins in vitro, using the pull-down assay. This would confirm the physical interaction between Lsb1 and Lsb2. First, we simultaneously overexpressed FLAG, HA-tagged Lsb2 with HA-tagged Lsb1 under the $P_{CUP1}$ copper inducible promoter, as well as the opposite orientation (FLAG, HA-tagged Lsb1 with HA-tagged Lsb2) in both wild type and *las17Δ* strains for 24 hours. Indeed, in both strains, we observed that pull-down of Lsb1 revealed the appearance of a band corresponding to Lsb2, and similarly, Lsb2 pull-down revealed Lsb1 protein, though much less in both experiments (Figure 3C). Moreover, we show that this interaction is not mediated by the Las17 protein, as the interaction between Lsb1 and Lsb2 is observed in the *las17Δ* strain. This allows us to demonstrate that there exists an interaction between the Lsb proteins independent of Las17.

To ensure our results were not merely an artifact of overexpression of these two

proteins, we performed pull-downs of HA-tagged Lsb1 and Lsb2 expressed from the endogenous chromosomal promoter. Once again, we confirm that Lsb2 forms an interaction with Lsb1 and that the reciprocal association is also observed (Figure 3D). This interaction is observed in spite of lower expression levels of both Lsb proteins.

**Lsb2 and Lsb1 are associated with cortical actin patches**

Given their ability to bind Las17 actin organizing protein, we sought to determine whether Lsb1 and Lsb2 associate with the actin cytoskeleton. Using fluorescently-tagged copies of each gene, we have found that Lsb1 and Lsb2 colocalize with actin patch marker protein Cap2-RFP, with the majority of Lsb1 and Lsb2 aggregates found near the periphery or next to the vacuole of the cell (Figure 3E). By mutating the conservative W residue in both Lsb1 (W90S) and Lsb2 (W91S), we abolish of the formation of punctate structures and aggregates associated with the actin patches and instead see the diffuse distribution of green fluorescence throughout the cytoplasm (Figure 3F). Thus, the Lsb proteins associate with the actin cortical patches in a manner dependent on the conserved W residue within the SH3 domain, which is also required for binding to Las17 and ubiquitin.

**Discussion**

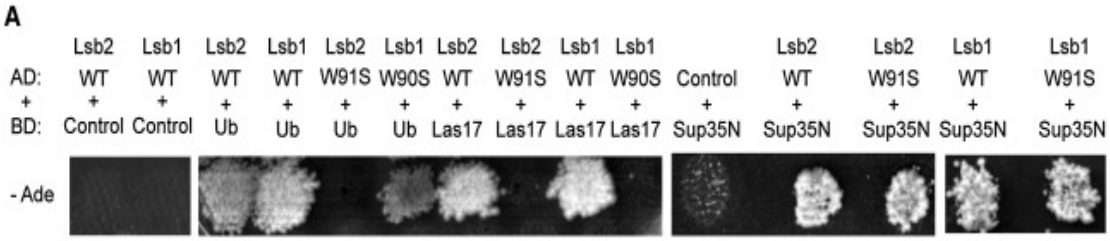
Overall, these data have allowed us to confirm preliminary findings of large scale analyses which have been performed on proteins in yeast, and add to our knowledge of the cellular associations of the Lsb proteins, which also bind to Sup35 and each other.

Of particular interest is the fact that Lsb2 interaction with both Ub and Las17 is mediated by the same residue in the N-terminal SH3 domain. This was initially predicted using a homology model to represent the possible interaction between Lsb2 and

ubiquitin, where we observed that the hydrophobic region of the SH3 domain would be presented towards the ubiquitin molecule and that, within this region, we located a tryptophan residue conserved in both Lsb2 (W91) and Lsb1 (W90). We show that this is indeed a site required for non-covalent ubiquitin binding, independent of the lysine residues which undergo ubiquitination (a covalent modification). Therefore, this conservative site could serve as a point of cross-talk between the ubiquitination of Lsb2 and its association with La17, and suggests that these are two competing, regulatory processes that may influence Lsb2 protein stability. Similarly, this mechanism of regulation may be conserved for Lsb1 protein.

Interaction of Lsb1 with Sup35 and Lsb2 suggest that Lsb1 could be involved or interfere with Lsb2 prion related function. In the future, it will be of interest to identify the regions implicated in the interaction of the Lsb proteins with Sup35, as this will allow us to analyze which regions of Lsb2 are fundamentally required for its prion inducing ability. By determining the nature of the Lsb1-Sup35 interaction, we can begin to predict its function in binding Sup35 and ascertain if this coincides with a possible role of the Lsb1 protein in the prion formation process via Sup35 or the prion-induction process via Lsb2.

We will also further analyze the significance and role of the Lsb2-Lsb1 interaction in yeast. It remains possible that Lsb1 protein affects the ability of Lsb2 to induce prions, whether by influencing the aggregation of Lsb2, influencing the stability of Lsb2 protein or through some other regulatory function.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Lsb2 W91S + Las17
Lsb1 WT + Las17
Lsb1 W90S + Las17
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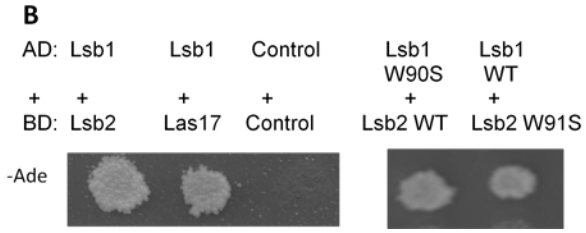
- Ade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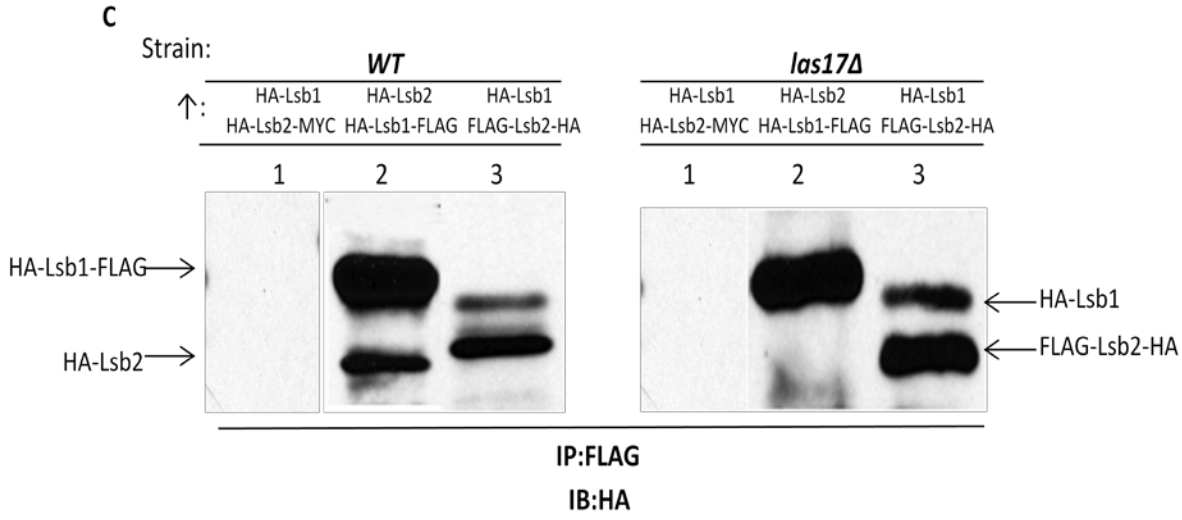
-Ade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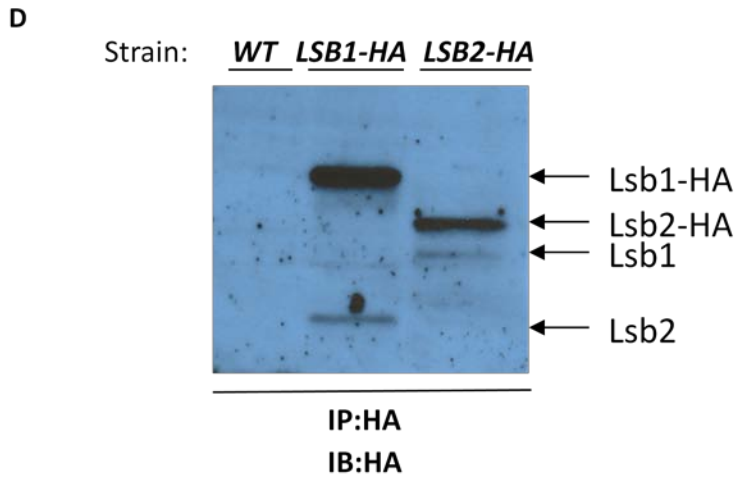
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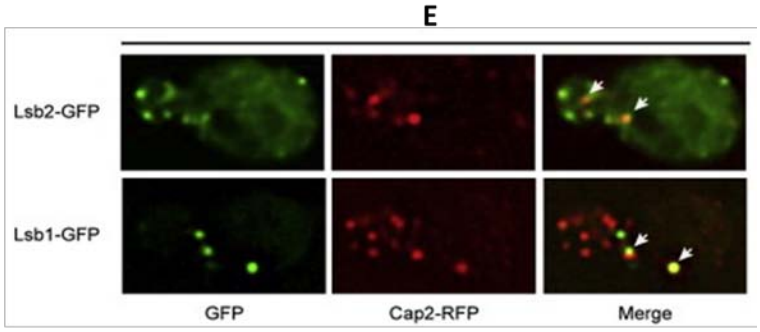
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Lsb1-GFP
GFP
Cap2-RFP
Merge

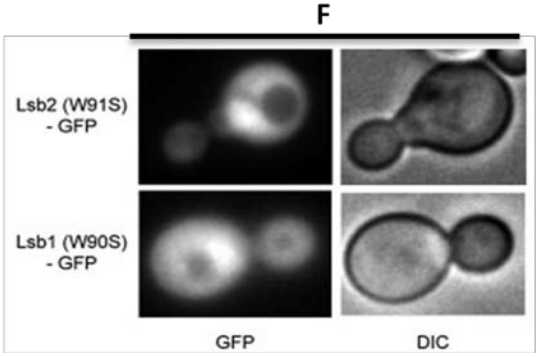

F
Lsb2 (W91S)
- GFP
Lsb1 (W90S)
- GFP
GFP
DIC

Figure 3. Protein binding partners of the Lsb proteins

(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 $P_{GAL}$-*ADE2* reporter construct, resulting in growth on −Ade (shown after 5 days of incubation). Results for the N-terminal PrD of Sup35, Sup35N (shown in the 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) Lsb1 and Lsb2 protein interaction is not mediated through conservative tryptophan residue in the N-terminal SH3 domain. (C) Pulldown experiments of FLAG and HA-tagged Lsb1 and Lsb2 proteins overexpressed under the $P_{CUP1}$ promoter for 18 hours in both wild type and *las17Δ* strains confirm physical interaction between Lsb1 and Lsb2 proteins. (D) Pulldowns of HA-tagged Lsb1 and Lsb2 expressed under the endogenous chromosomal promoter confirm physical interaction of Lsb1 and Lsb2 protein. (E) Colocalization of Lsb protein with a marker protein for actin patches, Cap2 (as indicated by arrows). (F) Substitution of the conserved tryptophan (W) residue in the SH3 domain of Lsb1 or Lsb2 abolishes formation of punctate and aggregated structures.

# Chapter 5 Influence of Las17 on the protein dynamics and stability of the Lsb proteins

## Introduction

Although we have identified Las17 as an interacting partner of the Lsb proteins, the role of this association remains uncharacterized. Given the function of Las17 in the organization of actin patches, it is possible that Lsb proteins are anchored to the cortical actin patches through their association with Las17. Indeed, when we mutate the conservative tyrosine (W) residue to serine (S) of either Lsb protein, we abolish their association with the actin cytoskeleton but also their ability to bind Las17. Here, we perform experimentation to further our understanding of the Lsb protein-Las17 interaction to reveal whether Las17 plays an essential role in the prion induction process via Lsb2 or whether it influences the possible regulatory function of Lsb1 within the process of prion formation.

## Results

### Presence of Las17 protein influences the Lsb protein stability

To determine the role of Las17 protein in influencing the stability of the Lsb proteins, we overexpressed HA-tagged Lsb1 and Lsb2 under the $P_{CUP1}$ promoter in wild type and *las17Δ* strains, and observed cellular levels of Lsb1 and Lsb2 in both logarithmic and stationary phase cells. We determine that, in the absence of Las17 protein, levels of Lsb1 within actively dividing cells (1-2 hours post induction) are significantly diminished compared to the wild type strain, but are measurable, possibly due to accumulation, during stationary phase (18 hours post induction) (Figure 4A). Similarly, we notice that Lsb2 protein levels are diminished, though not to the extent of

Lsb1 protein (Figure 4B). However, at all time points analyzed, the level of Lsb2 protein in the *las17Δ* strain is significantly reduced in comparison to levels in the wild type strain (Figure 4B).

**Inability to bind the actin cytoskeleton further compromises the Lsb protein stability**

We next sought to determine if the W90S mutation of Lsb1 and W91S mutation of Lsb2 within the N-terminal SH3 domain would impact the protein stability of the Lsb proteins. Overexpression of HA-tagged Lsb2 and Lsb1 mutants in wild type strain results in comparable levels of protein between both mutant and wild type Lsb proteins, although ubiquitinated conjugates of both Lsb proteins were reduced by the W mutation (Figure 4B). It is possible that the inability to bind Las17 does not necessarily influence the stability of either Lsb protein but that maintaining the presence of Las17 and its function in actin polymerization is required for the stability of Lsb proteins. In fact, when the Lsb-actin binding mutants are overexpressed in *las17Δ* strain, there is a further reduction in both Lsb2 and Lsb1 protein levels (Figure 4B). These experiments suggest that both the presence of Las17 protein and the ability of Lsb1 and Lsb2 to bind actin are necessary to maintain the protein stability of the Lsb proteins.

**Mutation of the major site of ubiquitination of Lsb1 and Lsb2 proteins restores protein levels**

In order to discern whether decreased levels of Lsb1/2 proteins in the absence of Las17 are the result of increased ubiquitination and degradation, we analyzed levels of mutant Lsb proteins, which lack the ability to undergo ubiquitination. By overexpressing HA-tagged, lysine mutant Lsb proteins (incapable of ubiquitination), we partially

stabilized levels of both Lsb1 and Lsb2 in the *las17Δ* strain, similar to levels observed in the wild-type strain (Figure 4C and Figure 4D, respectively).

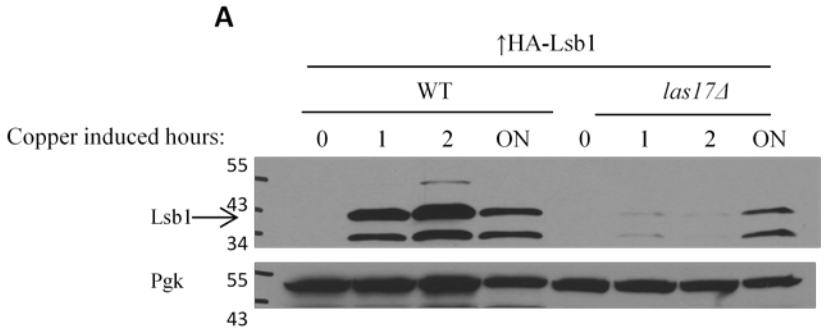
**Discussion**

By analyzing the expression of wild type and mutant Lsb proteins in the presence or absence of Las17 protein, we revealed the role Las17 may play in the process of Lsb protein stability and protein regulation in yeast. Furthermore, we were able to show that it is the functional presence of Las17 protein which is required for maintenance of steady-state levels of both Lsb proteins, not the direct interaction of Lsb1 and Lsb2 with Las17, as the cellular levels of the Lsb –actin binding mutants (W→S) are comparable to wild-type Lsb proteins in wild-type strain. We are also able to comment upon the role of actin binding of both Lsb2 and Lsb1, as this, in part, allows for Lsb2 and Lsb1 to remain present and functional within the cell. From our data, inability to bind actin leads to degradation of both Lsb proteins via a UPS-dependent pathway (possibly via the Cdc48-Ufd1-Npl4 complex, unpublished data 2011) and that, by preventing ubiquitination of the Lsb proteins, we are able to inhibit their degradation.

It has already been shown that the Lsb2 W91S mutation, unable to bind the actin cytoskeleton, is incapable of inducing [*PSI*$^+$] prion in yeast; however, the role of Las17 in this process has not been determined. Given its ability to influence Lsb protein stability, it will be of particular importance to study the effect of *las17Δ* on the ability of Lsb2 to induce [*PSI*$^+$] prion. It is possible that presence Las17 can serve as a protective mechanism to ensure the maintenance and accumulation of Lsb proteins, and allow Lsb2 to function in the prion induction process.

Here, we can further expand upon our hypothesis that binding to Las17 and Ub

can be two competing, regulatory processes that may influence Lsb1 and Lsb2 protein stability. Indeed, ubiquitination can serve the regulatory function to remove and degrade Lsb proteins that are bound to Las17. Presence of Las17 protein stabilizes the cellular levels of Lsb1 and Lsb2 and, when it is absent, ubiquitination is able to signal degradation of the Lsb proteins. By continuing our studies of the role ubiquitination and Las17 binding to the Lsb proteins, we can discover how these processes may influence the *de novo* formation of prions, regulating the formation and degradation of intracellular aggregates.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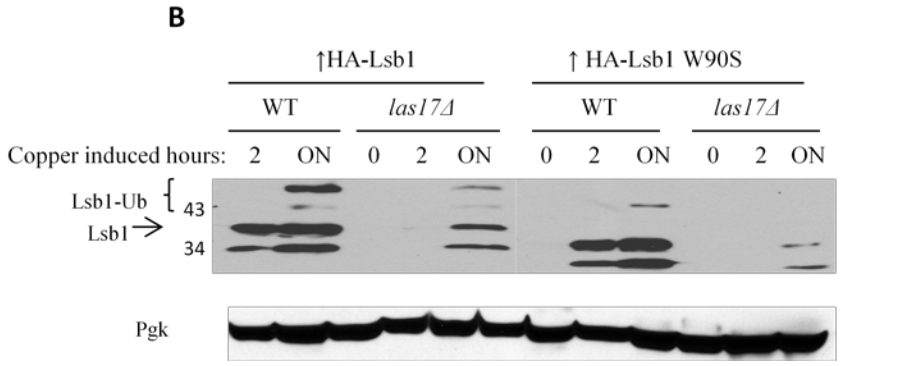
B
↑HA-Lsb1
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Lsb1
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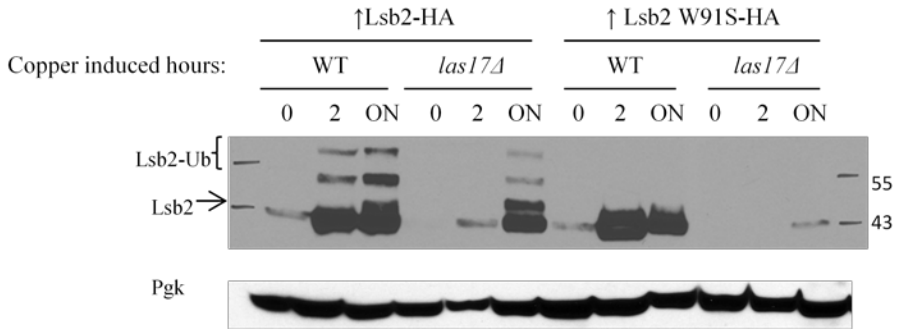
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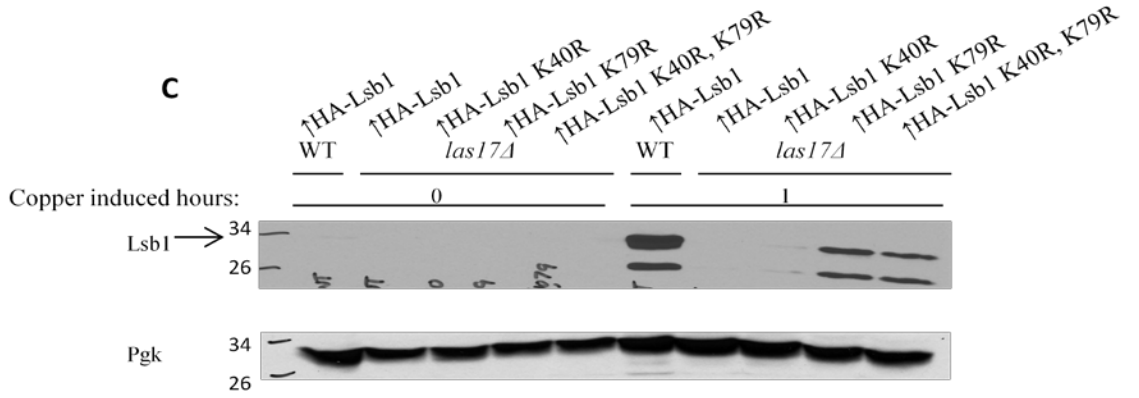
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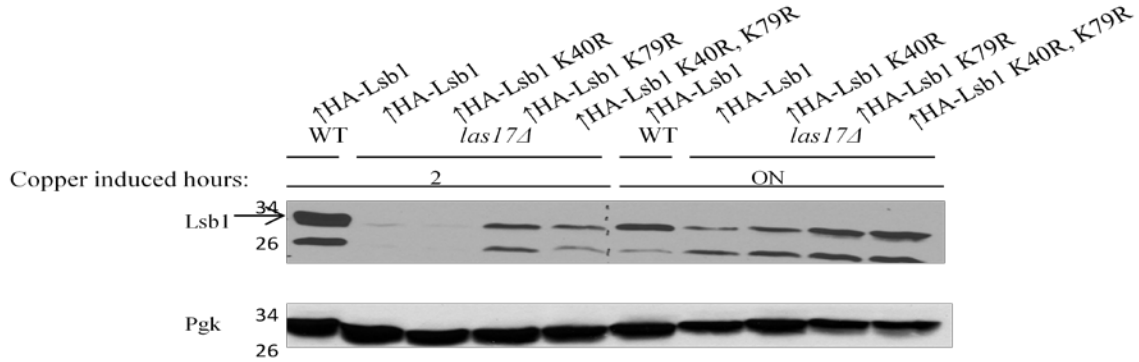
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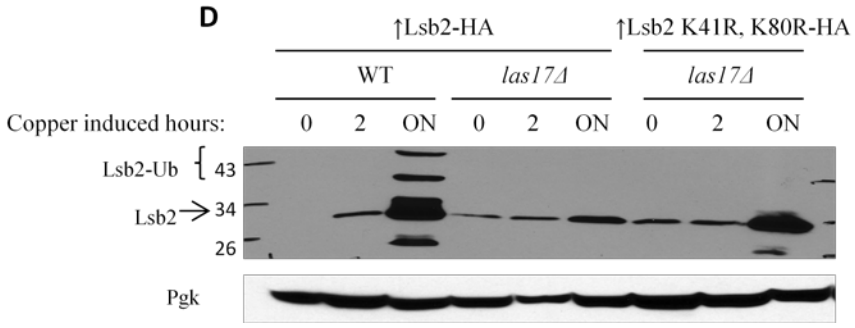
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Lsb2
34
26
Pgk

Figure 4. Lsb protein stability is influenced by *LAS17*, ubiquitination and association with actin patches

(A) Overexpression of HA-Lsb1 under the $P_{CUP1}$ promoter for indicated time points reveals decreased protein levels in *las17Δ.* (B) Overexpression of HA-Lsb1, HA-Lsb1 W90S, Lsb2-HA, and Lsb2 W91S-HA under the $P_{CUP1}$ promoter for indicated time points shows that the function of Las17, not association with Lsb proteins, influences cellular levels of Lsb1 and Lsb2. (C) and (D) Overexpression of ubiquitination mutants of HA-Lsb1 (K40R, K79R, K40R,K79R) (C) and Lsb2-HA (K41R,K80R) (D) under the $P_{CUP1}$ promoter restores cellular levels of Lsb proteins in *las17Δ* strain. Protein samples in (A-D) were detected using anti-HA Ab.

# Chapter 6 The Lsb proteins are processed within yeast

## Introduction

Protein processing events have been shown to be key regulators of protein activation in pathways ranging from the unfolded protein response to control of lipid metabolism (Wang et al., 1994). In addition, processing has been implicated in the activation of dormant precursor proteins, including the transcription factors SPT23 and MGA2, through an ubiquitin/proteasome-dependent pathway (Jentsch and Pyrowolakis, 2000). Here, we present evidence which shows that both Lsb proteins appear to be processed but that the site of processing is not conserved. We also hypothesize the effect of Lsb1 processing on impacting its potential ability to induce [$PSI^+$] prion. By studying the processing events of the Lsb proteins, we can begin to unlock the biological significance of their processing and what role it may play in the prion formation process.

## Results

### Lsb1 protein is processed at its C-terminal at adjacent tyrosine residues Y187, Y188

By overexpression of two HA-tagged constructs of Lsb1, HA-Lsb1 and Lsb1-HA, we detected the appearance of two bands through western blot analysis of HA-Lsb1 corresponding to full-length Lsb1 (top band) and the processed version of Lsb1 (lower band) (Figure 5A). The processed band of Lsb1 was not detected in cells where Lsb1-HA was overexpressed, supporting the notion that cleavage at the C-terminal would remove the HA epitope and prevent detection. In addition, we also observe Lsb1 processing when Lsb1 is expressed from the endogenous chromosomal promoter (Figure 5B). It appears that of the ratio of full-length to processed protein is 1:1. In addition, processing of Lsb1 does not appear to be dependent upon (or mediated by) the presence of Lsb2 protein, as

the expression of full-length and processed Lsb1 appear similar in both wild type and *lsb2Δ* strains. Additionally, after expressing N-terminal HA-tagged mutants of Lsb1, we show that both Lsb1 W90S and Lsb1 ubiquitin mutants are processed, revealing that binding to Las17 and the actin patches, as well as the ubiquitination process is not required for Lsb1 processing (Figure 4B and Figure 4C).

To determine the site of processing, overexpressed HA-Lsb1 protein was purified from yeast, and subjected to mass spectrometry analysis. Data revealed that Lsb1 is processed at either one or two adjacent tyrosine residues located within the C-terminal Q/N-rich domain, within a region that is conserved in both Lsb proteins. We next performed site-directed mutagenesis upon either one tyrosine or both tyrosines, substituting alanine (A) amino acid. Although mutation of Y187 resulted is a decreased amount of processed Lsb1, only mutation of both tyrosines, Lsb1 YY187188AA, abolished/prevented Lsb1 processing (Figure 5C). Nevertheless, further experimentation will be needed to determine the role of Lsb1 processing in yeast.

**Lsb2 protein is processed at its N-terminal**

Similar experiments were performed to determine if Lsb2 protein is also processed. We first performed overexpression studies of N-terminal HA-tagged Lsb2, but were only able to reveal the presence of full-length Lsb2. This suggested that Lsb2 was not processed at its C-terminal. However, overexpression of C-terminal HA-tagged Lsb2 revealed the appearance of both full-length Lsb2 (top-band) and processed Lsb2 (lower band), whereas previously, only the full-length version was detected by expressing N-terminal HA-tagged Lsb2 (Figure 5D). It is evident that processed Lsb2 represents much less of the total Lsb2 protein present, as we detect significant accumulation of processed

Lsb2 in stationary phase cell, though not in actively dividing cells. Additionally, after expressing C-terminal HA-tagged mutants of Lsb2, we show that both Lsb2 W91S and Lsb2 K41R, K80R mutants are both similarly processed, suggesting that binding to Las17 and the actin patches, as well as the ubiquitination process is not required for Lsb2 processing (Figure 5D).

Currently, the site of Lsb2 processing has not been determined but remains of particular interest as it may influence prion induction via Lsb2.

**Lsb1 C-terminal processing may prevent *de novo* [*PSI*$^{+}$] induction**

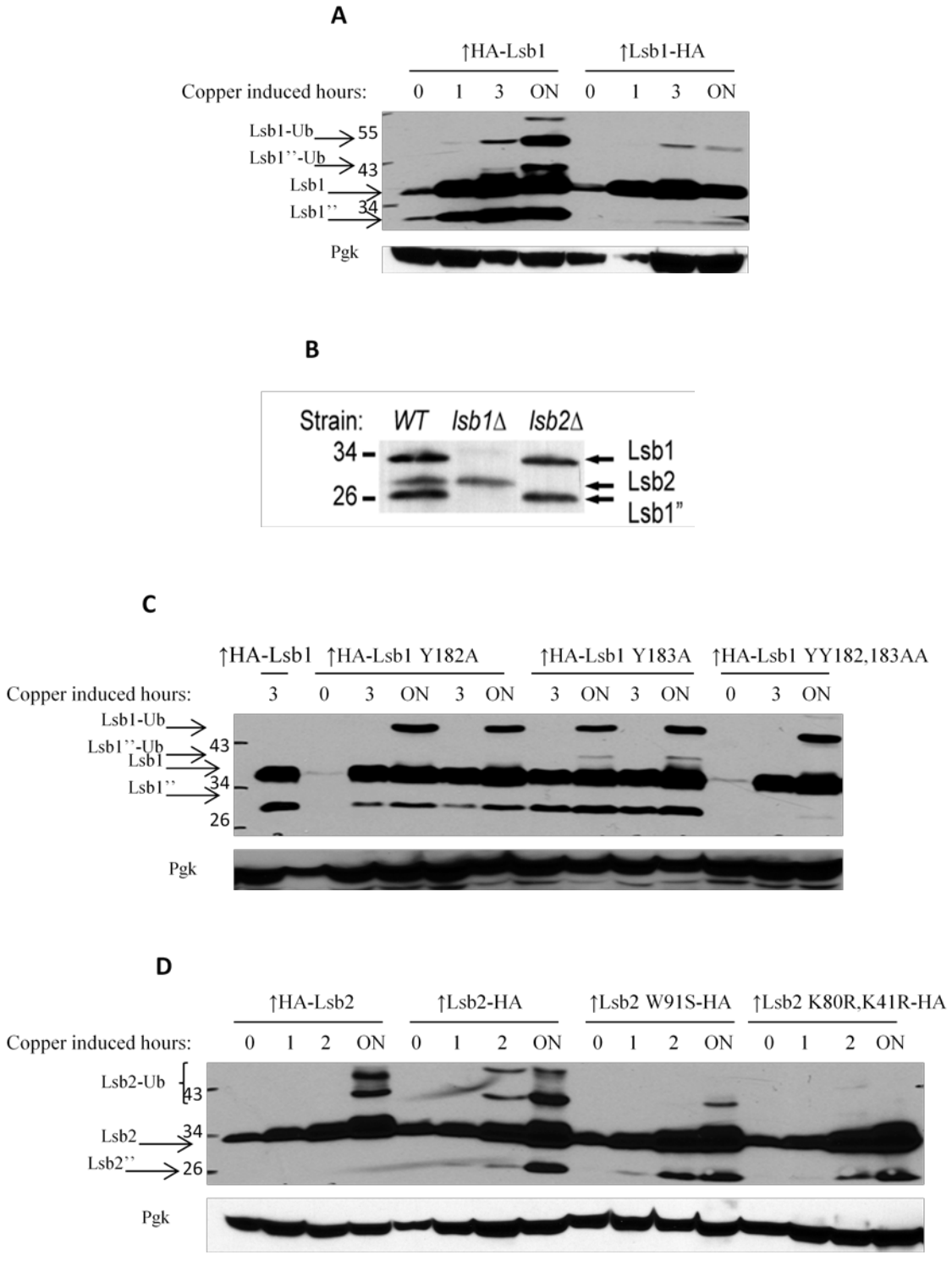
In our analysis of Lsb1 processing, we observed that Lsb1 is processed at a site directly adjacent to its Q/N-rich domain. We also report a relatively equal ratio of full-length to processed Lsb1 in the cell. Consequently, this event of Lsb1 processing would effectively lead to the separation of the N-terminal SH3 domain and the C-terminal Q/N-rich domain. It has previously been shown that both the N-terminal SH3 domain, the conserved W residue, and the Q/N-rich C-terminal domain are essential for prion induction by Lsb2 (Chernova et al., 2011). Therefore, if Lsb1 processing does in fact separate these two critical domains, this could serve as a likely explanation for the difference in prion inducing ability exhibited by the Lsb proteins. To analyze the capability of the SH3 domain and the Q/N-rich domain of Lsb1 to contribute to prion induction, we created chimera protein constructs. Here, we attempted to determine if the C-terminal Q/N-rich domain of Lsb1 could induce prions when fused to the N-terminal SH3 domain of Lsb2 and whether the N-terminal SH3 domain of Lsb1 fused to the C-terminal Q/N-rich domain of Lsb2 is sufficient for prion induction. Using the nonsense suppression assay, we show that substitution of the Lsb2 SH3 domain with the SH3

domain of Lsb1 doesn't affect the prion inducing ability of Lsb2. This indicates that the SH3 domain of Lsb1 is capable of contributing to prion induction. Alternatively, Lsb2 prion inducing ability is dependent upon it retaining a C-terminal Q/N-rich domain that cannot become processed and that when we substitute the C-terminal Q/N-rich domain of Lsb1, we prevent prion induction via Lsb2 (Figure 5E). Intriguingly, when we substitute the corresponding N-terminal SH3 domain of Lsb1 into Lsb2, Lsb2 still maintains its prion inducing ability. This suggests that although the N-terminal SH3 domain of Lsb1 can function in the prion induction process, full-length Lsb1 may be incapable of inducing prions, due to its processing at the C terminus.

**Discussion**

Future experiments will determine if prevention of Lsb1 processing will render the Lsb1 protein capable of inducing prions. Similarly, we will seek to understand the effect of preventing Lsb1 and Lsb2 processing and determine if this will impact prion induction via Lsb2. Through in depth analysis of the protein structure of both Lsb1 and Lsb2, we begin to more clearly reveal the mechanism of prion induction via Lsb2, as well as identify the key regions of the protein which are required for this function.

Along with understanding why the Lsb proteins are processed, we are also investigating which proteins and which particular pathways are responsible for Lsb protein processing and the regulation of this event. This, too, may have implications in the ability of the Lsb proteins to induce prions.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Pgk
B
Strain: WT lsb1Δ lsb2Δ
34
26
Lsb1
Lsb2
Lsb1"
C
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Copper induced hours:
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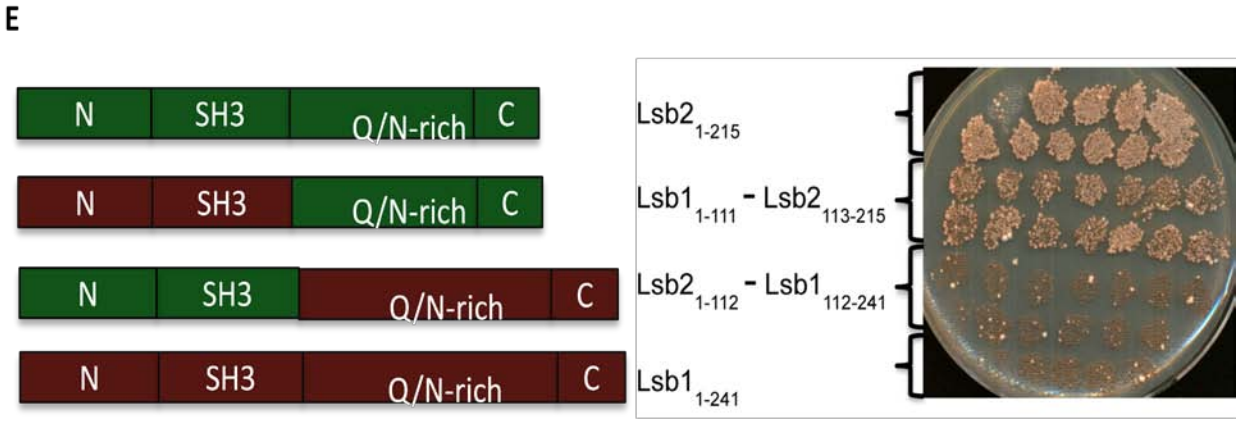



Figure 5. Analysis of Lsb protein processing

(A) Overexpression of HA-Lsb1 under the $P_{CUP1}$ promoter reveals full-length (top band) and processed (bottom band) Lsb1 protein, as well as their respective ubiquitin conjugates, showing that Lsb1 protein is processed at its C-terminal. (B) Expression of Lsb1-HA (detected using anti-Lsb2/Lsb1 Ab) under the endogenous chromosomal promoter confirms processing of Lsb1 protein. (C) Overexpression of mutant HA-Lsb1 protein, HA-Lsb1 YY182, 183AA, under the $P_{CUP1}$ promoter prevents processing of Lsb1 protein. (D) Overexpression of Lsb2-HA under the $P_{CUP1}$ promoter reveals full-length (top band) and processed (bottom band) Lsb2 protein, showing that Lsb2 protein is processed at its N-terminal. (E) Overexpression of wild type and chimera protein constructs, $Lsb2_{1-215}$, $Lsb1_{1-111}$-$Lsb2_{113-241}$, $Lsb2_{1-112}$-$Lsb1_{112-241}$, and $Lsb1_{1-241}$ along with Sup35 to induce [$PSI^{+}$] prions, as detected by growth on –Ade medium. N-terminal SH3 domain of Lsb1, but not the C-terminal Q/N-rich domain, can contribute to prion induction by Lsb2. Protein samples in (A), (C), and (D) were detected using anti-HA Ab.

# Chapter 7 Effects of Lsb1 protein on the prion induction process via Lsb2

## Introduction

Detection of [*PSI*$^+$] prion is performed using the nonsense suppression assay. When native Sup35 (eRF3) translation termination factor is present in yeast, it will recognize the premature stop codon (*UGA*) in the *ADE1* gene (*ade1-14*) (Chernoff et al., 2002). Subsequently, this strain will not be able to grow on medium lacking adenine (-Ade). However, upon adopting its prion form, partial loss of Sup35 function allows read-through and growth of the strain on -Ade medium. Confirmation that Ade+ colonies contain [*PSI*$^+$] prion is confirmed by showing that the Ade+ phenotype is curable by GuHCl.

Given our current knowledge of the interaction between the Lsb proteins, and the interaction of Lsb1 with Sup35, we sought to address the question as to whether Lsb1 protein plays a part in the prion induction process via Lsb2. To answer this question, we created *lsb1Δ* in strains used for *[PSI+]* prion detection and performed subsequent analysis of prion induction by overexpression of Lsb2 and Sup35 in this strain.

## Results

### Absence of *LSB1* enhances prion induction process via Lsb2

Disruption of the *LSB1* gene was generated by PCR-mediated gene replacement in [*psi*$^-$, *pin*$^-$] strains (Longtine et al., 1998). After simultaneous co-overproduction of Lsb2 and Sup35, as well as "sequential" overexpression, where Lsb2 is transiently overproduced before Sup35 overproduction, we detected [*PSI*$^+$] induction on –Ade media after expression of both proteins was arrested. We observe that in the *lsb1*Δ strain,

efficiency of prion induction is greatly enhanced, leading to an almost two-fold increase in abundance of [*PSI*$^{+}$]colonies detected (Figure 6A and 6B).

**Discussion**

Here, we show that in the absence of Lsb1 protein, the prion induction process via Lsb2 is still observed, suggesting that Lsb1 protein is not required for [*PSI*$^{+}$] induction. In fact, absence of Lsb1 enhances the prion inducing ability of Lsb2. Therefore, in the presence of Lsb1 protein, the prion induction process via Lsb2 may be impacted by the interaction documented between the Lsb proteins. As we have hypothesized, Lsb1 appears to serve some regulatory function within the process of prion formation and, based on our data presented here, could inhibit efficient prion formation via Lsb2. One possible explanation for this result is that Lsb1 binds and sequesters a particular population of Lsb2 or Sup35 protein, preventing it from participating in the prion induction process. Subsequently, when Lsb1 protein is no longer present, a larger fraction of Lsb2 is capable of functioning in the active formation of [*PSI*$^{+}$] prion. Further experimentation will explore specifically how Lsb1 negatively influences/regulates the prion induction process via Lsb2.

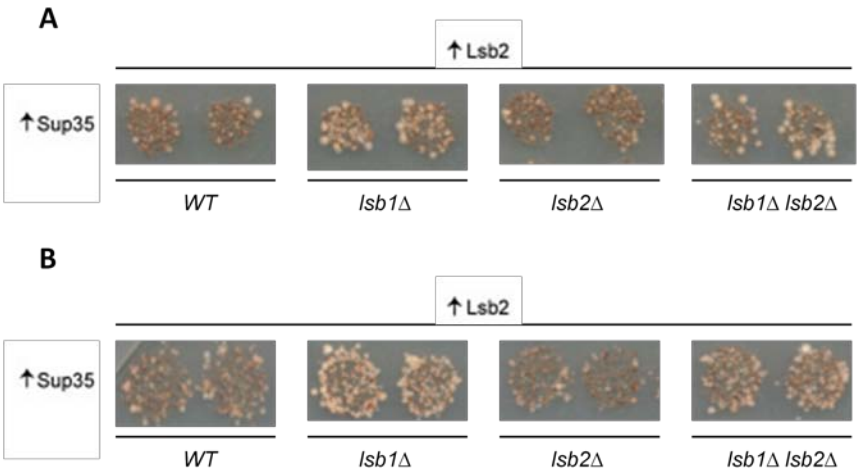



Figure 6. Efficiency of prion inducing ability of Lsb2 is influenced by absence of *LSB1* Overexpression of Lsb2 enables excess Sup35 to induce [*PSI*$^{+}$] in WT and *lsb2Δ* strains, as detected by growth on –Ade medium. Prion inducing ability of Lsb2 is enhanced in the absence of LSB1, as seen in both *lsb1Δ* and *lsb1Δlsb2Δ* strains. Observations are confirmed using simultaneous co-overproduction of Lsb2 and Sup35 (A) and sequential overproduction (B), where Lsb2 is transiently overproduced before Sup35 overproduction.

# Chapter 8 Role of the Lsb proteins in the maintenance of [*PSI*$^{+}$] prion

## Introduction

Based on our current understanding, Lsb2 directly induces the formation of *[PSI+]* via Sup35. In addition, our recent data support the hypothesis that Lsb1 modulates the prion induction process via Lsb2. Here, we observe what effect the absence of Lsb proteins has on the maintenance, or retention, of *[PSI+]* prion as a result of thermal stress. This is a separate but related process to prion formation in yeast; therefore, by uncovering a role for the Lsb proteins in the maintenance of prions, we can begin to discern the cellular function of Lsb1 and Lsb2 proteins.

## Results

### 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 and 39°C, respectively (Figure 7E and Figure 7A). Indeed, a transient shift to high temperature induced Lsb2 (Figure 7A and Figure 7B) to levels comparable to our artificial overexpression experiments (Figure 7B) and returned to pre-stress levels after 30–60 minutes at 39°C.

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 7C) (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 and loss of prion (Figure 7D). Neither wild-type nor *lsb2Δ* 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.

**Lsb1 protein influences [*PSI*$^+$] maintenance**

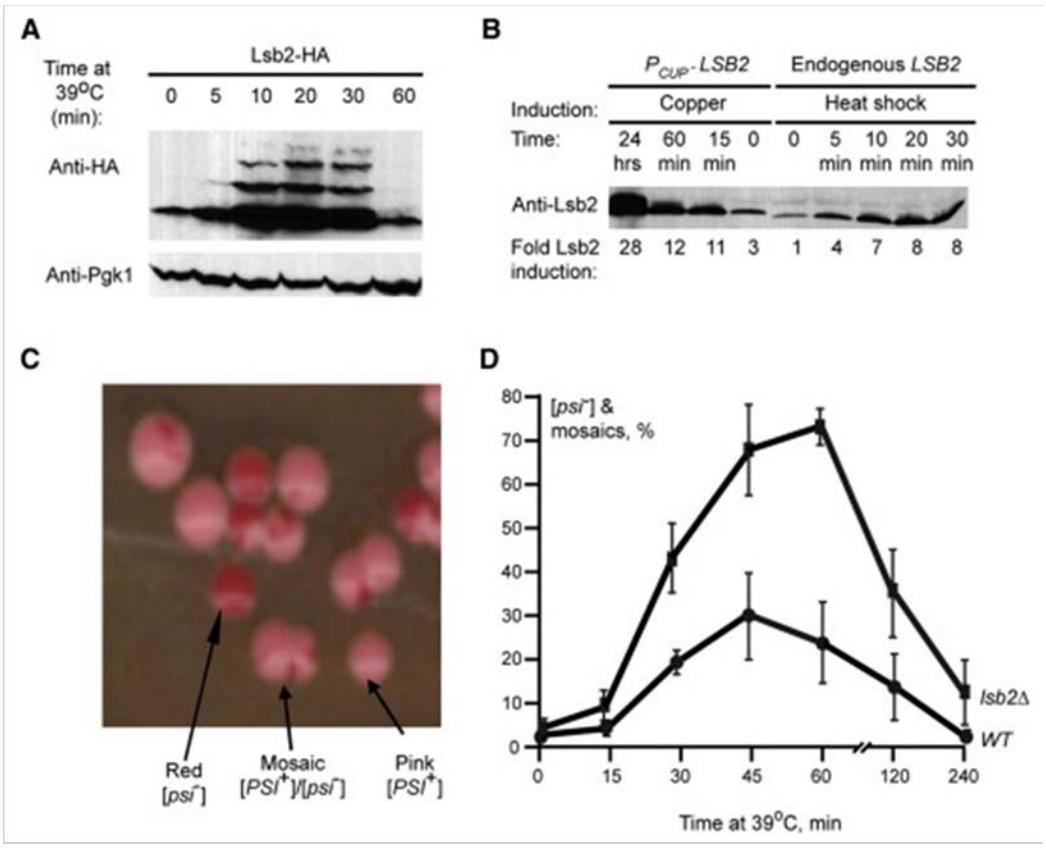
Similarly, we next attempted to characterize whether Lsb1 proteins achieved similar elevation in cellular levels as a result of stress. However, expression of HA-tagged endogenous Lsb1 remained steady throughout the time-course of heat shock at 37°C (Figure 7E), suggesting that levels of full-length Lsb1 protein are maintained and not subject to stress-inducible overexpression.

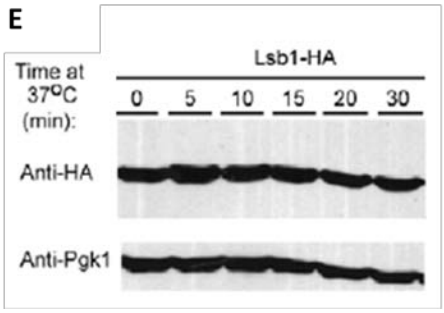
In order to determine if Lsb1 influences [*PSI*$^+$] during stress, and if this function is analogous to the observed role of Lsb2 in prion maintenance, we compared the effects of heat shock on [*PSI*$^+$] maintenance in wild-type, *lsb1Δ, lsb2Δ* and *lsb1Δ lsb2Δ* strains. We found that deletion of *LSB1* significantly increases heat-induced [*PSI*$^+$] destabilization and loss of prion (Figure 7F), akin to the effect observed upon deletion of *LSB2*. Here, absence of either Lsb1 or Lsb2 proteins results in a similar response of cells upon mild heat shock, resulting in elevated numbers of prion-cured colonies. Interestingly, double deletion of both *LSB1* and *LSB2* results in a reduction of heat-induced [*PSI*$^+$] destabilization, which closely mimics the trend observed in wild-type cells. Therefore, both Lsb1 and Lsb2 exhibit some similar role within process of prion maintenance during stress.

**Discussion**

The frequency of prion induction is dependent on the rates of *de novo* prion formation and the loss of prion from [*PSI*$^+$] 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 [*PSI*$^+$] variant after short-term heat shock, thus confirming the role of stress-induced Lsb2 in prion maintenance under these conditions.

In addition, we show that Lsb1 functions in a similar capacity to influence the maintenance of [*PSI*$^+$] under thermal stress conditions. This is one of the first experiments that have begun to reveal what role Lsb1 may play within the cell. It appears that Lsb1 and Lsb2 accomplish a similar function in the maintenance of [*PSI*$^+$] prion. Given the fact that both *lsb1Δ* and *lsb2Δ* strains behave similarly, it is possible that the two proteins serve a redundant role in the stability of [*PSI*$^+$] and that cells can perform this function with one or the other of these proteins. However, in the absence of both proteins, cells may adapt a compensatory pathway that allows them to partially restore the [*PSI*$^+$] state, thus preventing the loss of prion. These experiments reveal the possibility that, in the maintenance of [*PSI*$^+$], the Lsb proteins may play a conserved role and that, in response to loss of the Lsb proteins function, cells are selected to recruit additional mechanisms which significantly enhance [*PSI*$^+$] stability.


A
Time at 39°C (min):
Lsb2-HA
0 5 10 20 30 60
Anti-HA
Anti-Pgk1
B
P CUP - LSB2
Endogenous LSB2
Induction:
Copper
Heat shock
Time:
24 hrs 60 min 15 min 0
0 5 min 10 min 20 min 30 min
Anti-Lsb2
Fold Lsb2 induction:
28 12 11 3 1 4 7 8 8
C
Red [psi-]
Mosaic [PSI+]/[psi-]
Pink [PSI+]
D
[psi-] & mosaics, %
80 70 60 50 40 30 20 10 0
lsb2Δ
WT
0 15 30 45 60 120 240
Time at 39°C, min


E
Time at 37°C (min):
Lsb1-HA
0 5 10 15 20 30
Anti-HA
Anti-Pgk1

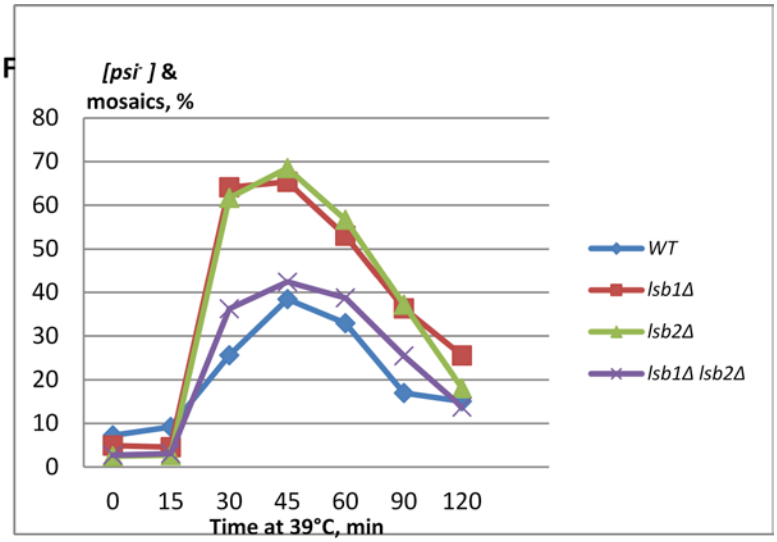



Figure 7. Lsb Levels and Effects during Stress

(A) Thermal stress induces Lsb2. Pgk1 protein was used as a loading control. (B) Levels of Lsb2 induction by copper and heat shock. Protein levels were analyzed at indicated time points using Lsb2 Ab. (C) [*psi*$^{-}$] colonies are induced by heat shock. (D) [*PSI*$^{+}$] destabilization and recovery during heat shock in *WT* and *lsb2Δ* strains. (E) Thermal stress does not induce Lsb1. (F) [*PSI*$^{+}$] destabilization and recovery during heat shock in *WT*, *lsb1Δ, lsb2Δ*, and *lsb1Δlsb2Δ* strains. For (C), (D), and (F), yeast were grown to early exponential stage at 25°C, shifted to 39°C for specified period of time, and then plated on YPD and incubated at 25°C. Error bars correspond to standard deviations.

# Chapter 9 Discussion and Future Directions

We began studies of the Lsb proteins with the knowledge that Lsb2 protein was identified in a large-scale screen as an inducer of [*PSI*$^+$], while its close paralog Lsb1 was not identified in this study (Derkatch et al., 2001). Nevertheless, after beginning examination of the role of Lsb2 protein in [*PSI*$^+$] prion induction, we found it beneficial to investigate properties of its paralog, Lsb1, which would serve as a valuable control allowing us to analyze specific characteristics of both proteins that contribute to and/or prevent from promoting the process of prionogenesis. What we have uncovered is that despite minor sequence divergence between the Lsb proteins, only Lsb2 protein promotes *de novo* formation of [*PSI*$^+$] prion. Surprisingly, we find that Lsb1 protein may influence the prion inducing ability of Lsb2 through a regulatory mechanism that is currently unclear. In contrast, both Lsb1 and Lsb2 proteins appear to influence the maintenance of [*PSI*$^+$] prion under stress conditions. Combined, these observations provide preliminary indications that Lsb1 protein may play a key function in the prion induction and prion maintenance processes via Lsb2, illustrating some of the possible biological relevance of Lsb proteins.

## *Interactions of Lsb1 and Lsb2 in Prion Formation*

Based on our previous findings, we have proposed that increases in levels of Lsb2, caused by heat shock and other stresses, can trigger the accumulation of misfolded Sup35 at the cytoskeleton-associated cortical regions. Indeed, we have shown that the W91S mutation of Lsb2 that prevents association with the actin cytoskeleton abolishes its prion inducing activity (Chernova et al., 2011). This highlights the importance of Lsb2

protein ability to bind to the actin cytoskeletal machinery. Cytoskeletal networks would then target aggregated Sup35 to the quality control compartments adjacent to the vacuole. This partly protects the [*PSI*$^{+}$] prion from uncontrolled agglomeration and elimination during short-term heat shock. In [*psi*$^{-}$] cells, the increased local concentration of Lsb2 and Sup35 could then facilitate prion formation (Figure 9).

Notably, the levels of Lsb2 protein accumulate during heat stress and are similar to the levels causing prion induction, while the cellular levels of Lsb1 remain uninduced during heat stress (Figures 7A, 7B and 7E). Thus, Lsb2 accumulation in response to physiological stresses could be a trigger that induces formation of prions. Furthermore, failure of the UPS (e.g., during severe stress) should stabilize Lsb2 levels, thus exacerbating its potential prion-inducing effect. Previously, it has been demonstrated that mutation of the Lsb2 ubiquitination site caused Lsb2 accumulation and increased prion formation (Chernova et al., 2011). Here, we observe that Lsb1 is ubiquitinated in a manner similar to Lsb2 protein and that alterations of UPS also act to stabilize Lsb1 protein levels (Figures 2A, 2B and 2C). Therefore, the action of UPS on regulating cellular levels of Lsb1 and Lsb2 protein, in combination with the effects of environmental stress on induction of Lsb2 protein, may modulate the formation of [*PSI*$^{+}$]. The presence of Las17 protein appears to stabilize the Lsb proteins, and Las17 binding to Lsb2 and Lsb1 is, in part, mediated by the same site that is required for Lsb protein binding to Ub. Thus the levels of Ub may compete for Las17 binding and may provide an additional mechanism to modulate [*PSI*$^{+}$] formation (Figures 4A-4D and 3A).

In addition to the effects on prion formation, we show that Lsb1 and Lsb2 proteins interact directly using various detection systems (Figures 3B, 3C and 3D).

Indeed, it appears that the direct association of the Lsb proteins (i.e. interaction of Lsb1 and Lsb2 proteins) has a significant effect in influencing the dynamics and efficiency of prion formation and prion maintenance. In the induction of [*PSI*$^+$] prion via Lsb2, Lsb1 protein seems to behave as a negative regulator of Lsb2, preventing it from achieving its maximal ability to efficiently induce prions (Figures 6A and 6B). In part, this may be explained by the Lsb1 interaction with Lsb2, which reduces the levels of Lsb2 protein available to promote Sup35 protein aggregation and prionogenesis. It has been shown that the extent of spontaneous [*PSI*$^+$] prion formation is enhanced in a concentration-dependent manner. Therefore, Lsb1 protein may act by directly reducing the available Lsb2 protein pool. Another possibility is that Lsb1 interaction with Lsb2 occupies a region/domain of Lsb2 that is necessary for binding to Sup35 to induce the formation of prion aggregates. Thus, Lsb1-Lsb2 interaction may compete with the ability of free Lsb2 to bind Sup35 and/or associate with the actin cytoskeleton.

## *Effects of Lsb proteins on Prion Maintenance*

With respect to maintenance of [*PSI*$^+$] prion, deletion of either *LSB1* or *LSB2* appears to result in the destabilization of weak [*PSI*$^+$] under mild-heat shock conditions (Figures 7D and 7F). This suggests that both Lsb1 and Lsb2 proteins function in a similar manner to promote the retention of [*PSI*$^+$] prion. Moreover, given that Lsb1 and Lsb2 proteins associate in the cell, the maintenance of [*PSI*$^+$] may be a result of an Lsb protein (Lsb1-Lsb2) complex. Due to their association with the actin cytoskeleton, and specifically the actin cortical patches, the Lsb1-Lsb2 complex may allow for effective targeting and transport of [*PSI*$^+$] aggregates to daughter cells, resulting in retention of [*PSI*$^+$] in the growing cell population (Figure 3F). Loss of either protein manifests itself

in a similar outcome: the mutant cells are no longer as capable as wild type cells to prevent loss of [*PSI*$^{+}$] prion (Figure 8).

Surprisingly, in the combined absence of *LSB1* and *LSB2*, the ability to maintain [*PSI*$^{+}$] is completely restored, which could be the result of some response measure adapted by cells to overcome loss of Lsb1 and Lsb2 function. Further experimentation is necessary to support the model of the role of Lsb1 and Lsb2 interaction in yeast as it pertains to prion induction and prion maintenance of [*PSI*$^{+}$].

Here, loss of *LSB1* may interfere with association and transport of [*PSI*$^{+}$] aggregates from mother to daughter cells, via Lsb2 bound to the actin cytoskeleton machinery. Thus, not only may Lsb2 protein induce [*PSI*$^{+}$] prion formation, but, in addition, its association with the actin cytoskelton may play a role in the propagation of prions. Furthermore, since Lsb1 and Lsb2 proteins bind each other, maintaining a balance of both Lsb proteins in the cell may be necessary to regulate the function of Lsb2 protein in the processes of prion formation and maintenance.

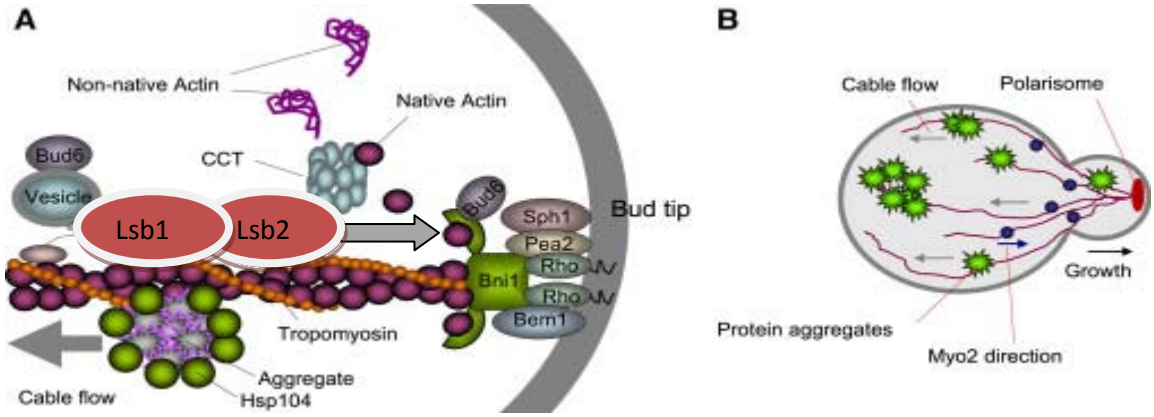
## *Processing of Lsb proteins*

A notable difference between Lsb1 and 2 is the processing observed with Lsb1. Approximately half of Lsb1 is cleaved at the dityrosine motif separating the SH3 and prion-like domain of Lsb1. Lsb2 is also processed, albeit at a much lower frequency and at a different site. It remains unclear what role processing of Lsb1 and Lsb2 proteins has within the cell (Figures 5A, 5B and 5D). In regards to Lsb1, we have identified the site of processing and are now beginning studies to determine what effect mutation of the adjacent tyrosine residues at the processing site has on the localization of Lsb1 protein. We will also assess if this mutation of Lsb1 will interfere with the interaction between

Lsb1 and Lsb2. In addition, it was observed that the processing recognition site present in the Lsb1 coding sequence is conserved in the amino acid sequence of Lsb2. Although Lsb2 appears to be processed at its N-terminal it is not likely to be important in the regulation of prionogenesis given the much lower fraction of Lsb2 that is processed. The amino acid sequence surrounding the YY site is absolutely conserved in Lsb2, and we hope to investigate why Lsb1 and Lsb2 proteins are differentially processed at this conserved site. We hypothesize that the tertiary structure differences between Lsb2 and Lsb1 may be responsible for enabling Lsb1, but not Lsb2, to be processed at this site.

## *Summary*

Based on our data, Lsb2 protein appears to function as a stress-dependent [$PSI^{+}$] prion inducer in yeast. The ability of yeast cells to convert from the [$psi^{-}$] to [$PSI^{+}$] state may provide a selective advantage to ensure survival when faced with certain environmental stresses, such as severe fluctuations in temperature, which may also influence prion propagation. When considering that the Lsb proteins play a role in the maintenance of [$PSI^{+}$] prion and that Lsb1 and Lsb2 proteins associate in the cell, the heat-shock mediated prion destabilization observed upon loss of either *LSB1* or *LSB2* may be a result of alteration of the proper balance of these proteins. Therefore, physiologically relevant variations in the abundance of these proteins (e.g. during environmental stresses) may disrupt the biological processes in which both Lsb1 and Lsb2 proteins function.




Adapted from *Liu et al., 2009*. Cell.

Figure 8. Model for Segregation and Retrograde Transport of Protein Aggregates

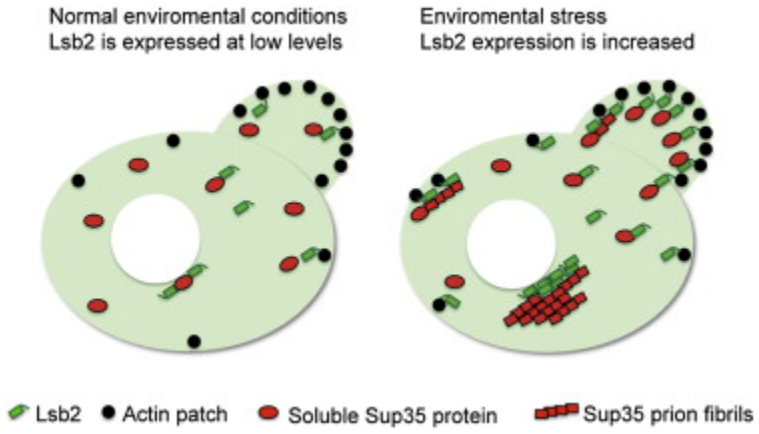



Figure 9. Model for the Stress-Dependent Induction of Sup35 Prions

# Chapter 10 Materials and Methods

### *Yeast Strains*

Disruptions and tagged derivatives of yeast chromosomal genes were generated by PCR-mediated gene replacement (Longtine et al., 1998). Coding sequences of yeast genes were PCR-amplified from *S. cerevisiae* genomic DNA or a template plasmid.

### *Yeast Plasmids*

The *LSB2* and *LSB1*coding sequences were cloned into pmCUP1 (Serio et al., 1999) under *PCUP1* promoter and *LSB2* was cloned in *CEN URA3* under *PGAL* promoter (Chernova et al., 2003) using the 5' primer introducing HA-tag at the gene N-terminus. For [*PSI+*] formation pLA1- CENGAL-Sup35 (HIS) and pFL39-CEN-GAL-Sup35N (TRP) (Chernova et al., 2003)and corresponding empty vectors were used. For colocalization analysis *LSB2* and *LSB1*coding sequence were cloned into pRS316 CG GFP (Serio et al., 1999) under *PCUP1* promoter to make a C-terminal fusion with GFP coding sequence. For yeast two hybrid analysis *LSB2, LSB1, LAS17* and Ub coding sequence were cloned into yeast two hybrid vectors pGBDU and pGAD (James et al., 1996) and analyzed along with pGBD-Sup35, pGBD-Sup35N and pACTII-Sup35N (Ganusova et al., 2006). Gene mutations were generated with the QuickChange Site-Directed Mutagenesis Kit from Stratagene and verified by sequencing.

***Protein Analysis***

For analysis of cellular levels of proteins (SDS-PAGE), overnight cell cultures were diluted and grown to OD~1.5. Cells from 1.5 mL of culture were collected and lysed by boiling in SDS-containing loading buffer. Overexpression analyses were performed within the indicated strains grown to logarithmic phase (OD~ 0.8-1.0), and protein expression was induced with 100 uM copper sulfate. Samples were obtained before induction (0 time-point), and at the indicated time-points post-induction. Protein extracts were examined by western analysis using specific antibodies and detection with the ECL detection system from Pierce. We used the following specific antibodies: anti-HA HA.11 (Covance, Inc., Emerville, California), anti-Pgk (Molecular Probes, Inc., Eugene, OR) as a loading control, anti-Lsb2 generated by ProSci, Inc., (Poway, Ca). In all experiments, we used appropriate secondary antibodies from GE Healthcare Ltd (Buckinhamshire, UK). Cycloheximide chase experiments to determine half-life of the Lsb proteins were conducted by Nela Moffat as described (Katzmann and Wendland, 2005).

***Heat Shock Experiments to determine Effects of Heat Shock on [PSI$^+$] loss (curing) and on cellular levels of Lsb proteins***

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 (0, and cells were lysed by boiling and analyzed by western blotting. For [*PSI*$^+$] curing experiments, aliquots were collected at the specified time points, plated onto YPD medium, and incubated at 25°C for 3-4 days (Newnam et al., 2011). [*PSI*$^+$] (light pink), [*psi*$^-$] (red), and mosaic [*PSI*$^+$]/[*psi*$^-$] colonies were detected

and calculated by visual inspection. Analysis of heat shock effects on endogenous cellular levels of Lsb1-HA and Lsb2-HA expression was conducted by Tatiana Chernova.

***Plate Assay for [PSI$^+$] Induction and Visualization***

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 (−Ade) (Chernoff et al., 2002). Individual transformants containing plasmids with *SUP35* under $P_{GAL1}$ and *LSB2* under $P_{CUP1}$ promoters were patched on synthetic medium (SD) selective for both plasmids and then replica plated either onto galactose (Gal) medium with $CuSO_4$ (Cu) for simultaneous induction of both proteins. For sequential induction, these cells were plated 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 20 independent transformants were tested for each strain-plasmid combination; the majority (or all) showed the same result in each case.

***Fluorescence Microscopy***

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

***Pulldown Experiments***

We simultaneously overexpressed FLAG, HA-tagged Lsb2 with HA-tagged Lsb1, both under the $P_{CUP1}$ copper inducible promoter, as well as the opposite orientation (FLAG, HA-tagged Lsb1 with HA-tagged Lsb2) in both wild type and *las17Δ* strains for 24 hours. After breaking cells by vortexing with glass beads, we incubated the cell lysate with FLAG M2 affinity gel (Sigma-Aldrich) for 18 hours at 4℃. Following overnight incubation, the resin was washed four times with Protein Lysis Buffer (without protease inhibitors), and bound proteins were eluted by the suspension of resin in SDS loading buffer and boiling for 5 minutes at 100℃. Finally, samples were examined via western analysis.

For pulldowns of endogenous Lsb1-HA and Lsb2-HA, cells were grown to logarithmic phase before cells were lysed by vortexing with glass beads. With the exception of utilizing anti-HA agarose (Thermo Scientific), subsequent steps were performed mirroring the protocol used in the pulldowns experiments of overexpressed Lsb1 and Lsb2.

***Yeast Two-Hybrid Analysis***

Two-hybrid experiments employed the [*psi*$^{-}$] strain PJ69-4A, containing the $P_{GAL}$-*ADE2* reporter constructs (James et al., 1996). For this analysis, we introduced wild type *LSB2* and *LSB1* genes into the Gal4-DNA Activating Domain (pGAD) vector, with Ub, Las17, Sup35 and Sup35N (N-termimal PrD domain only) being fused to the Gal4-DNA Binding Domain (pGBDU vector) (James et al., 1996). Point mutations of *LSB2* and *LSB1* genes were generated by QuikChange Site-Directed Mutagenesis protocol

(Stratagene). “Control” refers to plasmids bearing either $Gal4_{DBD}$ (pGBDU) or $Gal4_{ACT}$ (pGAD) domains without an insert, respectively. Activation of the $P_{GAL}$-*ADE2* reporter construct resulting from a two-hybrid interaction leads to growth on −Ade medium, shown after 5 days of incubation.

| Strain | Genotype | Source |
|---|---|---|
| **GT81-1C** | ***MAT*a *ade1–14 (UGA) his3-Δ200 leu2–3,112 lys2–801, trp1–289 ura3–52* [*PSI*$^+$] [*RNQ*$^+$]** | **Chernoff et al., 2000** |
| **GT409** | [*psi*$^-$][*pin*$^-$] derivative of GT81-1C | Allen and Chernova et al., 2006 |
| **WTY357** | *lsb2Δ::kanMX6* disruptant of GT 409 | Chernova et al., 2011 |
| **WTY730** | *lsb1Δ::HIS3MX6* disruptant of GT409 | Ali, Moiez |
| **WTY731** | *lsb2Δ::kanMX6 lsb1Δ::HIS3MX6* disruptant of GT409 | Ali, Moiez |
| **GT229** | *las17Δ::HIS3MX6* disruptant of GT81-1C | Bailleul, Peggy |
| **MHY501** | *MATα his3-Δ200 leu2–3,112 lys2–801, trp1–1 ura3–52* | Chen et al., 1993 |
| **WTY623** | *lsb2Δ::kanMX6* disruption of MHY501 | Chernova et al., 2011 |
| **WTY659** | *lsb1Δ::HIS3MX6* disruption of MHY501 | Ali, Moiez |
| **WTY660** | *lsb2Δ::kanMX6 lsb1Δ::HIS3MX6* disruption of MHY501 | Ali, Moiez |
| **WTY664** | *LSB2::3HA- kanMX6* insertion of MHY 501 | Chernova et al., 2011 |
| **WTY666** | *LSB1::3HA- kanMX6* insertion of MHY 501 | Chernova et al., 2011 |
| **WTY565** | *LSB2::GFP(S65T)- kanMX6* insertion of MHY 501 | Chernova et al., 2011 |
| **WTY663** | *CAP2::RFP-HIS3MX6* insertion of MHY 501 | Ali, Moiez |
| **MHY3646** | *MATα doa3-1 his3-Δ200 leu2–3,112 lys2–801, trp1–1 ura3–52* | Chen et al., 1993 |
| **WTY665** | *LSB2::3HA- kanMX6* insertion of MHY 3646 | Chernova et al., 2011 |
| **WTY669** | *LSB1::3HA- kanMX6* insertion of MHY 3646 | Chernova et al., 2011 |
| **FW1808** | MATα*, rsp5-1 his4-912δR5, lys2-128δ, ura3-52* | Huibregtse et al., 1997 |
| **FY56** | MATα*, his4-912δR5, lys2-128δ, ura3-52* | Huibregtse et al., 1997 |
| **OT55** | *MAT***a**, *ade1-14* (UGA) *his3-Δ200 leu2-3, 112 ura3-52, lys2–801 trp1–289* [*PSI*$^+$] [*RNQ*$^+$] | Newnam et al., 1999 |
| **WTY682** | *lsb2Δ::kanMX6* disruption of OT55 | Ali, Moiez |

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|---|---|---|
| **WTY 732** | *lsb1Δ::HIS3MX6* disruption of OT55 | Ali, Moiez |
| **WTY 733** | *lsb2Δ::kanMX6* *lsb1Δ::HIS3MX6* disruption of OT55 | Ali, Moiez |

| Name | Vector | Gene | Source |
|---|---|---|---|
| **WTD 115** | pmCUP | HA-LSB2 | Chernova et al., 2011 |
| **WTD 188** | pmCUP | LSB2-HA | Ali, Moiez |
| **WTD 144** | pmCUP | HA-LSB2 QQ174,175AA | Chernova et al., 2011 |
| **WTD 211** | pmCUP | HA-LSB2 QQQQ172,173,174,175AAAA | Ali, Moiez |
| **WTD 189-A** | pmCUP | LSB2 K40R,K80R-HA | Ali, Moiez |
| **WTD 195** | pmCUP | LSB2 W91S-HA | Emory DNA CCCF |
| **WTD 128** | pmCUP | HA-LSB1 | Chernova et al., 2011 |
| **WTD 171** | pmCUP | LSB1-HA | Ali, Moiez |
| **WTD 133** | pmCUP | HA-LSB1 K41R | Chernova et al., 2011 |
| **WTD 132** | pmCUP | HA-LSB1 K79R | Chernova et al., 2011 |
| **WTD 134** | pmCUP | HA-LSB1 K41R,K79R | Chernova et al., 2011 |
| **WTD 135** | pmCUP | HA-LSB1 PP135,136AA | Chernova et al., 2011 |
| **WTD 181** | pmCUP | HA-LSB1 W90S | Ali, Moiez |
| **WTD 198** | pRS316 | HA-LSB1 Y182A | Emory DNA CCCF |
| **WTD 199** | pRS316 | HA-LSB1 Y183A | Emory DNA CCCF |
| **WTD 200** | pRS316 | HA-LSB1 YY182, 183AA | Emory DNA CCCF |
| **WTD 189-B** | pRS315 | HA-LSB2-MYC | Emory DNA CCCF |
| **WTD 187** | pRS315 | HA-LSB1-FLAG | Emory DNA CCCF |
| **WTD 190** | pRS315 | FLAG-LSB2-HA | Emory DNA CCCF |
| | pRS316 CG | LSB2-GFP | Chernova et al., 2011 |
| **WTD 145** | pRS316 | LSB1-GFP | Chernova et al., 2011 |
| **WTD 137** | pRS316 | LSB2 W91S-GFP | Chernova et al., 2011 |
| **WTD 147** | pRS316 | LSB1 W90S-GFP | Chernova et al., 2011 |
| **WTD 185** | pRS316 | $LSB1_{1-111}$-$LSB2_{113-215}$ | Emory DNA CCCF |
| **WTD 186** | pRS316 | $LSB2_{1-112}$-$LSB1_{112-241}$ | Emory DNA CCCF |
| **GT#465** | pFL39 | SUP35 | Chernova et al., 2011 |
| **WTD 160** | pGAD | LSB2 | Ali, Moiez |
| **WTD 155** | pGAD | LSB1 | Ali, Moiez |
| **WTD 164** | pGAD | LSB2 W91S | Ali, Moiez |
| | pGAD | CONTROL (LEU) | James et al., 1996 |
| | pGBDU | CONTROL (URA) | James et al., 1996 |
| **WTD 166** | pGBDU | UBIQUITIN | Ali, Moiez |
| **GT#236** | pGBDU | SUP35N | Chernova et al., 2011 |
| **WTD 141** | pGBDU | LSB2 | Chernova et al., 2011 |
| **WTD 154** | pGBDU | LAS17 | Chernova et al., 2011 |

## Chapter 11 References


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