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Investigation

Innate immunity to yeast prions: Btn2p and Cur1p curing of the [URE3] prion is prevented by 60S ribosomal protein deficiency or ubiquitin/proteasome system overactivity

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Abstract

[URE3] is an amyloid-based prion of Ure2p, a negative regulator of poor nitrogen source catabolism in *Saccharomyces cerevisiae*. Overproduced Btn2p or its paralog Cur1p, in processes requiring Hsp42, cure the [URE3] prion. Btn2p cures by collecting Ure2p amyloid filaments at one place in the cell. We find that *rpl4aΔ*, *rpl21aΔ*, *rpl21bΔ*, *rpl11bΔ*, and *rpl16bΔ* (large ribosomal subunit proteins) or *ubr2Δ* (ubiquitin ligase targeting Rpn4p, an activator of proteasome genes) reduce curing by overproduced Btn2p or Cur1p. Impaired curing in *ubr2Δ* or *rpl21bΔ* is restored by an *rpn4Δ* mutation. No effect of *rps14aΔ* or *rps30bΔ* on curing was observed, indicating that 60S subunit deficiency specifically impairs curing. Levels of Hsp42p, Sis1p, or Btn3p are unchanged in *rpl4aΔ*, *rpl21bΔ*, or *ubr2Δ* mutants. Overproduction of Cur1p or Btn2p was enhanced in *rpn4Δ* and *hsp42Δ* mutants, lower in *ubr2Δ* strains, and restored to above wild-type levels in *rpn4Δ ubr2Δ* strains. As in the wild-type, Ure2N-GFP colocalizes with Btn2-RFP in *rpl4aΔ*, *rpl21bΔ*, or *ubr2Δ* strains, but not in *hsp42Δ*. Btn2p/Cur1p overproduction cures [URE3] variants with low seed number, but seed number is not increased in *rpl4aΔ*, *rpl21bΔ* or *ubr2Δ* mutants. Knockouts of genes required for the protein sorting function of Btn2p did not affect curing of [URE3], nor did inactivation of the Hsp104 prion-curing activity. Overactivity of the ubiquitin/proteasome system, resulting from 60S subunit deficiency or *ubr2Δ*, may impair Cur1p and Btn2p curing of [URE3] by degrading Cur1p, Btn2p or another component of these curing systems.

Keywords: UBR2; RPL4A; RPL21B; RPN4

Introduction

Most of the prions (infectious proteins) of *Saccharomyces cerevisiae* are self-propagating amyloids, each a linear polymer of a single protein with an in-register parallel folded β-sheet architecture (reviewed in Liebman and Chernoff 2012; Wickner *et al.* 2015). [URE3] is an amyloid-based prion of the Ure2 protein (Wickner 1994; Masison and Wickner 1995; Edskes *et al.* 1999; Brachmann *et al.* 2005), a repressor of genes for utilization of poor nitrogen sources (Cooper 2002). [PSI+] is an amyloid-based prion of Sup35p (Wickner 1994; Glover *et al.* 1997; King *et al.* 1997; Paushkin *et al.* 1997; King and Diaz-Avalos 2004; Tanaka *et al.* 2004), a subunit of the translation termination factor (Stansfield *et al.* 1995; Zhouravleva *et al.* 1995). [PIN+], a prion of Rnq1p (function unknown), is detected by its ability to rarely prime [PSI+] formation (Derkatch *et al.* 1997, 2001; Sondheimer and Lindquist 2000). Most variants of [PSI+] and [URE3] prions are either lethal or cause severely slowed growth (Mcglinchey *et al.* 2011), but even the mild variants of each (such as those used in most lab experiments) are rare in wild strains (Nakayashiki *et al.* 2005), demonstrating that even these are detrimental (Kelly *et al.* 2012). In fact, there have not yet been reproducible benefits of any variant of either prion (reviewed in Wickner *et al.* 2015). Thus, it is not surprising that, through the action of a series of anti-prion systems, normal cells are found to eliminate most variants of [URE3] and of [PSI+] as they arise(reviewed by Wickner *et al.* 2018; Son and Wickner 2020).

Among these anti-prion systems, normal levels of Btn2p and Cur1p cure a large majority of [URE3] variants arising in their absence (Kryndushkin *et al.* 2008; Wickner *et al.* 2014). In general, those [URE3] variants with low-propagation/seed numbers are cured by normal levels of Btn2p or Cur1p, while [URE3] variants with higher seed number, such as [URE3-1], require overproduction of Btn2p or Cur1p to eliminate the prion (Kryndushkin *et al.* 2008; Wickner *et al.* 2014). Normal levels of Btn2p and Cur1p measurably lower the propagon number of [URE3-1] although they cannot cure this variant (Kryndushkin *et al.* 2008). Overproduced Btn2p collects Ure2p amyloid filaments at one location in the cell, co-incident with Btn2p itself (Kryndushkin *et al.* 2008; Kanneganti *et al.* 2011). It is proposed that this sequestration of prion filaments explains the frequent loss of the prion as cells

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divide (Kryndushkin *et al.* 2008). Sites in Ure2p outside the prion domain affecting curing of [URE3] by overproduced Btn2p may identify regions of interaction with Btn2p (Shailesh *et al.* 2020). Btn2p also binds to and collects other amyloids and other non-amyloid aggregates, but does not cure the variants of [PSI+] or [PIN+] that have been tested (Kryndushkin *et al.* 2008, 2012; Malinovska *et al.* 2012; Barbitoff *et al.* 2017). Btn2p also cures an artificial prion based on a domain of Nrp1p (Malinovska *et al.* 2012). Cur1p, although paralogous to Btn2p, does not co-localize with the amyloid filaments during curing and may act by a different mechanism (Kryndushkin *et al.* 2008; Barbitoff *et al.* 2017).

Studying an artificial prion, in cells grown at 37°C, in the presence of the proteasome inhibitor MG132, Malinovska *et al.* (2012) found that overexpression of Sis1p prevented prion curing by overexpressed Btn2p or overexpressed Cur1p. They proposed that Btn2p and Cur1p cure prions by sequestration in the nucleus of Sis1p, an Hsp40 necessary for the propagation of the [URE3], [PSI+], and [PIN+] prions (Higurashi *et al.* 2008). However, this proposal explains neither Btn2p's co-localization with Ure2p prion amyloid (Kryndushkin *et al.* 2008), nor the retention of [URE3]-curing activity after deletion of the Btn2p nuclear localization sequence (Kryndushkin *et al.* 2008), nor the curing of 90% of [URE3] variants by the normal levels of Btn2p or Cur1p (Wickner *et al.* 2014), which are 20- and 400-fold (respectively) below that of Sis1p (https://pax-db.org, Wang *et al.* 2015). Moreover, although Sis1p is necessary for the propagation of the [URE3], [PSI+] and [PIN+] prions (Higurashi *et al.* 2008), neither [PSI+] nor [PIN+] was cured by Btn2p or Cur1p overproduction (Kryndushkin *et al.* 2008). Perhaps the overproduced Sis1p binds to and prevents the curing action of Btn2p or Cur1p, the reverse of the hypothesized mechanism. Nonetheless, *overproduced* Cur1p may cure by the suggested Sis1p removal mechanism (Barbitoff *et al.* 2017).

Btn2p has another, apparently unrelated, role as a late endosome mediator of protein sorting (Chattopadhyay *et al.* 2003; Kama *et al.* 2007, 2011; Kanneganti *et al.* 2011). *BTN2* was first identified as a gene overexpressed in strains deleted for *BTN1*, the yeast homolog of the Batten's disease gene (*CLN3*) (Chattopadhyay *et al.* 2000). In *btn2*Δ strains, Yif1p is mislocalized to the vacuole and Kex2p to the late endosome (Chattopadhyay *et al.* 2003; Kama *et al.* 2007). Btn2p co-localizes with Snx4p and Vps27p, two endosome markers, and immunoprecipitates with endocytic SNARE proteins Snc1p, Snc2p, Tlg1p, Tlg2p, and Vti1p (Kama *et al.* 2007). Kama *et al.* (2007) have shown that the protein sorting function of Btn2p requires at least Snc1p, Tlg2p, Vps26p, Snx4p, Ypt6p, Chs4p, and Rhb1p, but whether the same components are involved in the prion-curing activity of Btn2p is unknown. In a two-hybrid screen with Btn2p as bait, Btn3p was detected (Kanneganti *et al.* 2011). Overproduced Btn3p is evenly distributed in the cytoplasm, but is concentrated in discrete loci with overproduced Btn2p, binds directly to Btn2p, and inhibits both the protein-sorting and prion-curing activities of Btn2p (Kanneganti *et al.* 2011). Btn3p is necessary for normal endosomal sorting of ubiquitylated cargos and endosomal recycling of the Snc1 SNARE, even without Btn2p overproduction (Morvan *et al.* 2015).

Here, we report a screen for co-factors whose mutation prevents curing of [URE3] by overproduced Btn2p or Cur1p. Our screen utilizes the HERMES transposon, originally from house flies, but adapted for use in *S. cerevisiae* (Gangadharan *et al.* 2010; Guo *et al.* 2013), and utilized for the discovery of genes preventing [URE3] prion pathology (Edskes *et al.* 2018). We find that mutation of 60S ribosomal subunit protein genes or *UBR2*, encoding an E3 ubiquitin protein ligase, substantially impair curing activities of Btn2p and Cur1p. We examine the mechanism of these effects. We also tested seven of the proteins involved in the protein sorting functions of Btn2p, and, remarkably, none were necessary for the prion curing activity of either Btn2p or Cur1p.

Materials and methods

Strains and media

Strains of *S. cerevisiae* are listed in Table 1. Media are described by Sherman (1991). The reduced—adenine medium ½ YPD is 5 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, and 20 g/l agar and is used for scoring the [URE3] phenotype. *DAL5* transcription is strongly repressed by Ure2p in the presence of ammonia or other good nitrogen source (Cooper 2002), and we use a *DAL5*-promoted *ADE2* to measure Ure2p activity, and thereby, the presence or absence of [URE3] (Schlumpberger *et al.* 2001; Brachmann *et al.* 2005). [URE3] strains are white or pink, while [ure-o] strains are red on ½ YPD. Synthetic complete and dropout medium contained 6.67 g/l Yeast Nitrogen Base without amino acids (Difco), glucose (2%, dex) or galactose (3%) and raffinose (2%) (gal/raf), and an appropriate dropout mix from Sunrise Science (*e.g.*, 1.92 g/L of SC-W). All incubations were at 30°C unless noted otherwise.

Plasmid construction

Plasmids used are listed in Table 2.

pBEE103 (*CUP1* promoter—*SUP35NM CEN TRP1*) was constructed from pSL1066 (*CUP1* promoter—*SUP35NM CEN URA3*) by homologous recombination in yeast. *Stu*I-treated pSL1066 (cutting inside *URA3*) and a PCR product containing *TRP1* and regions of homology upstream and downstream of the *URA3* marker in pSL1066 were transformed into yeast selecting for Trp+. The *TRP1* PCR product was obtained using primers BEE3065 (5'CGCATCTGTGCGGTATTTCACACCGCATAGaacgacattactatatatat3') and BEE3066 (5'GCAGATTGTACTGAGAGTGCACCATACCACcaggcaagtgcacaaacaat3') and pRS314 as template. The resulting pBEE103 containing *TRP1* instead of *URA3* was used to make pBEE34 (*CUP1* promoter—*BTN2 CEN TRP1*) and pBEE42 (*CUP1* promoter—*CUR1 CEN TRP1*) by homologous recombination of pBEE103 digested by *Msc*I (cutting inside *SUP35NM*) with PCR products containing the *BTN2* or *CUR1* ORFs flanked by sequences in pBEE103 just upstream and downstream of the *SUP35NM* ORF. The PCR product for creation of pBEE34 was obtained using primers BEE3067 (5'GTACAATCAATCAATCAATCAGGATCCACAATGTTTTCCA TATTCAATTC3') and BEE3068 (5'TACGACTCACTATAGGGCGAATTGGAGCTCTTATATCTCCTCAATAATAG3') and pBEE1 as template. The PCR product for creation of pBEE42 was obtained using primers BEE3069 (5'GTACAATCAATCAATCAATCAGGATCCACAATGGCTGCCGCATGCATTTG3') and BEE3070 (5'TACGACTCACTATAGGGCGAATTGGAGCTCTTACCGCCCATTCAATCTTC3') and pBEE2 as template.

The *CEN LEU2 BTN3* plasmid pBEE92 was constructed from *Pst*I, shrimp alkaline phosphatase-treated pRS315 to which was ligated a *Pst*I-treated *BTN3-HA* construct including 562 bp 5' of the start codon. The *BTN3-HA* PCR product was from genomic DNA of BY241 using primers BEE3389 (5' ccctgcagCCGCAGCCTTGATTCTGCCTCGGG3') and BEE3390 (5' ccctgcagTTAAGCGTAATCTGGAACATCGTATGGGTAAGAAAGGATGGTGGCGTCG3').

To make pBEE95 (*CEN LEU2 HSP42* promoter (587 bp) *HSP42-HA*), a PCR product obtained using primers BEE3391 (5' ccc tgcagCACTTACAATTTAGGCCGCGCC3') and BEE3392 (5' ccc tgcagTCAAGCGTAATCTGGAACATCGTATGGGTAATTTTCTACCGTAGGGTTGGG3') was digested with *Pst*I and ligated to *Pst*I and shrimp alkaline phosphatase—treated pRS315.pBEE1 (*GAL1-*

Table 1 Strains of *Saccharomyces cerevisiae*

Strain	Parent	Genotype	Source
1735	MA116-8A	MATα *his- ura2-60 [URE3-1]*	(Lacroute 1971; Aigle and Lacroute 1975)
BEE1027	74D-694	MAT**a** *ura3 leu2 trp1 his3 ade1-14* [PIN+] [psi-]	(Chernoff *et al.* 1995)
BEE1021	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1* [URE3-1]	(Brachmann *et al.* 2005)
4591		MATα *his3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1* [ure-o]	(Brachmann *et al.* 2005)
BEE1190	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 hsp42::KanMX* [URE3-1]	(Wickner *et al.* 2014)
BEE1192	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 ubr2::KanMX* [URE3-1]	This study
BEE1251	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl21b::KanMX* [URE3-1]	This study
BEE1272	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl4a::KanMX* [URE3-1]	This study
BEE1318	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 ubr2::KanMX rpn4::URA3* [URE3-1]	This study
BEE1363	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl16b::KanMX* [URE3-1]	This study
BEE1364	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl11b::KanMX* [URE3-1]	This study
BEE1372	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rps14a::KanMX [URE3-1]*	This study
BEE1378	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rps30b::KanMX* [URE3-1]	This study
BEE1086	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 snc1::KanMX* [URE3-1]	This study
BEE1096	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 snc2::KanMX* [URE3-1]	This study
BEE1132	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 tlg2::KanMX* [URE3-1]	This study
BEE1125	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 vps27::KanMX* [URE3-1]	This study
BEE1219	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 vps35::KanMX* [URE3-1]	This study
BEE1088	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 vps26::KanMX* [URE3-1]	This study
BEE1012	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 btn2::TRP1 cur1::kanMX* [URE3-1]	(Kryndushkin *et al.* 2008)
BEE1182	BY245	MATα *his3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1* [ure-o]	This study
BEE1200	5169-3D	MATα *trp1 kar1 DAL5p-ADE2 leu2 ura3 Hsp104T160M—URA3* [ure-o]	This study
BEE1201	5169-7B	MATα *trp1 kar1 DAL5p-ADE2 leu2 ura3* [ure-o]	This study
BEE1202	5168-9A	MATα *trp1 kar1 DAL5p-ADE2 leu2 ura3 Hsp104T160M—URA3* [ure-o]	This study
BEE1203	5169-5A	MATα *trp1 kar1 DAL5p-ADE2 leu2 ura3* [ure-o]	This study
BEE1407	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl21a::KanMX* [URE3-1]	This study
BEE1319	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpn4::KanMX* [URE3-1]	Emily Stroobant and Herman Edskes
BEE1103	779-6A	MATα *kar1 ade2.1 SUQ5 his3Δ202 leu2Δ1 trp1Δ63 ura3.52* [PSI+ strong]	
BEE1104	779-6A	MATα *kar1 ade2.1 SUQ5 his3Δ202 leu2Δ1 trp1Δ63 ura3.52* [PSI+ Sc4]	Mike Reidy
BEE1105	779-6A	MATα *kar1 ade2.1 SUQ5 his3Δ202 leu2Δ1 trp1Δ63 ura3.52* [PSI+ Sc37]	Mike Reidy
BEE1106	779-6A	MAT**a** *kar1 ade2.1 SUQ5 his3Δ202 leu2Δ1 trp1Δ63 ura3.52* [PSI+ LiebW]	Mike Reidy
BEE1107	779-6A	MAT**a** *kar1 ade2.1 SUQ5 his3Δ202 leu2Δ1 trp1Δ63 ura3.52* [PSI+ LiebS]	Mike Reidy
BEE1386	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-CAN1: CAN1 kar1 arg1::TRP1 lys2::LEU2* [URE3-1]	Morgan DeWilde and Herman Edskes
BEE1387	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-CAN1: CAN1 kar1 arg1::TRP1 lys2::LEU2 hsp42::KanMX* [URE3-1]	This study
BEE1392	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-CAN1: CAN1 kar1 arg1::TRP1 lys2::LEU2 rpl4a::KanMX* [URE3-1]	This study
BEE1395	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-CAN1: CAN1 kar1 arg1::TRP1 lys2::LEU2 rpl21b::KanMX* [URE3-1]	This study
BEE1401	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-CAN1: CAN1 kar1 arg1::TRP1 lys2::LEU2 rps30b::KanMX* [URE3-1]	This study
BEE1271	BY241	MAT**a** *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl4a::KanMX* [ure-0]	This study
BEE1368	BY241	MATα *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl4a::KanMX* [ure-0]	This study
BEE1370	BY241	MATα *ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1* [ure-0]	This study
BEE1417	BY241		This study

(continued)

Table 1 (continued)

Strain	Parent	Genotype	Source
		MATa ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 rpl21b::KanMX rpn4::URA3 [URE3-1]	
5991	BY241	*MATa ura3 leu2 trp1 P-DAL5: ADE2 P-DAL5: CAN1 kar1 tma10::kanMX*	This study
74-D694		*MATa kar1 ade1-14 his3 leu2 trp1 ura3* [psi-][PIN+]	(Chernoff *et al.* 1995)
6010	BY241	*MATα ura3 leu2 trp1 his3::TRP1 P-DAL5: ADE2 P-DAL5: CAN1 kar1*	This study

Strain 1735 (MA116-8A) (Lacroute 1971; Aigle and Lacroute 1975) was used as cytoduction donor to BY241 BY241 (Brachmann *et al.* 2005) forming BEE1021. Segregants of crosses 5168 and 5169 were produced by crossing a strain carrying *hsp104-T160M-URA3* (Hung and Masison 2006) twice with BY241 producing strains BEE1200 to BEE1203.

Table 2 Plasmids

Plasmid	Promoter	Gene	Marker	Copy number	Source
pBEE34	*CUP1*	*BTN2*	*TRP1*	CEN	This study
pBEE42	*CUP1*	*CUR1*	*TRP1*	CEN	This study
pBEE95	*HSP42*	*HSP42-HA*	*LEU2*	CEN	This study
pBEE92	*BTN3*	*BTN3-HA*	*LEU2*	CEN	This study
pSG36	*GAL1*	*Hermes* transposase & *Hermes-natMX6 transposon*	*URA3*	CEN	(Gangadharan *et al.* 2010)
pBEE28	*GAL1*	*BTN2-RFP*, pYES52-BTN2-RFP	*URA3*	2 μm	(Kryndushkin *et al.* 2008)
pBEE29	*URE2*	*URE2N-GFP*, pVTG12	*LEU2*	CEN	(Edskes *et al.* 1999)
pBEE64	*GAL1*	*RPL4A*	*LEU2*	CEN	This study
pSL1066	*CUP1*	*SUP35NM-GFP*	*URA3*	CEN	(Zhou *et al.* 2001)
pRS314	none	empty vector	*TRP1*	CEN	(Sikorski and Hieter 1989)
pH316	*GAL1*	empty vector	*LEU2*	CEN	(Edskes and Wickner 2002)
pRS315	none	empty vector	*LEU2*	CEN	(Sikorski and Hieter 1989)
pBEE1	*GAL1*	*BTN2*	*LEU2*	CEN	(Zhao *et al.* 2018)
pBEE2	*GAL1*	*CUR1*	*LEU2*	CEN	(Zhao *et al.* 2018)
pBEE103	*CUP1*	*SUP35NM-GFP*	*TRP1*	CEN	This study
pET28-BTN2-HIS	*T7*	*BTN2-HIS*	*Kanamycin*		Shailesh Kumar, NIDDK
pET28-CUR1-HIS	*T7*	*CUR1-HIS*	*Kanamycin*		Shailesh Kumar, NIDDK
pH381	*GAL1*	*Ure2 1-89*	*LEU2*	CEN	(Edskes and Wickner, 2000)
p1289	*BTN2*	*BTN2*	*URA3*	2 μm	(Kryndushkin *et al.* 2008)
p1488	*CUR1*	*CUR1*	*URA3*	2 μm	(Wickner *et al.* 2014)
pRS426	none	empty vector	*URA3*	2 μm	(Christianson *et al.* 1992)
P1678	*GAL1*	*TMA10*	*HIS3*	CEN	This study

All plasmids are *E. coli*—*S. cerevisiae* shuttle plasmids except for pET28-BTN2-HIS and pET28-CUR1-HIS.

promoted *BTN2 CEN LEU2*) was constructed by inserting *Bam*HI-*Pst*I-treated PCR product with the *BTN2* coding sequence into *Bam*HI-*Pst*I-treated plasmid pH316 (*LEU2 CEN GAL1* promoter, Edskes and Wickner 2002). The PCR product with *BTN2* was made from p1475 (Kryndushkin *et al.* 2008) using primers: ATAGGATCCATGTTTTCCATATTCAATTC (BTN2BamHIF) and ATACTGCAGTTATATCTCCTCAATAATAG (BTN2PstIR) (Zhao *et al.* 2018). pBEE2 was constructed by inserting a *Bam*HI-*Hind*III-treated PCR product with the *CUR1* coding sequence into *Bam*HI-*Hind*III-treated plasmid pH316 (Edskes and Wickner 2002). The PCR product with *CUR1* was obtained using p1488 as template and primers: ATAGGATCCATGGCTGCCGCATGCATTTG (CUR1BamHIF) and GCGAAGCTTTTACCGCCCATTCAATCTTC (CUR1HindIIIR).pBEE64 (*GAL1* promoter-*RPL4A CEN LEU2*) was constructed by *in vivo* recombination in yeast substituting the *RPL4A* ORF for the *BTN2 ORF* in pBEE1 using a PCR product carrying *RPL4A* flanked by pBEE1 sequences upstream and downstream of *BTN2*. This PCR product was obtained using DNA from strain BEE1021 as template and primers BEE3334 (5'GTTAATATACCTCTATACTTTAACGTCAAGGAGAAAAAACCCC GGATCCATGTCCCGTCCACAAGTTAC3') and BEE3335 (5'CTTGA TTGGAGACTTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTCA GCTGCTGCAGTTAATCGTGTTTCAAAGTTT3').

Antibodies used

See Table 3.

Western blots

Yeast cells in log phase from 50 ml of synthetic complete media were collected, washed 3 times in 1 ml of water and resuspended in 600 μl of lysis buffer containing 8 M urea, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM Pefabloc SC, 1x complete EDTA-free protease inhibitor tablet (Pierce), 5% glycerol, 1xPBS and placed in 2 ml tubes with screw caps. Glass beads (0.5 mm) were added to fill the tubes and samples were homogenized at 4 C for 3 min using a Bead Beater (BioSpec) at maximum shaking parameters. Using a hot needle, a hole was made at the bottom of the 2 ml tube which was placed then inside a 5 ml tube with screw cap containing a hole in the cap which fits the 2 ml tube. Samples were centrifuged for 5 minutes at 3000 g at 4°C. The supernatant was placed in 1.5 ml tubes and centrifuged at maximum speed in a microcentrifuge. The supernatant was transferred to a fresh 1.5 ml tube, protein measured by BCA (Pierce) and stored at −80°C. Protein concentrations of samples for acrylamide gel electrophoresis were equalized, and samples were made 1x LDS sample buffer (ThermoFisher Scientific), and 175 mM DTT. After

Table 3 Antibodies used

Specificity	Animal	Titer	Source
Anti-Btn2 polyclonal	Rabbit	1:5,000	custom generated by COVANCE
Anti-Cur1 polyclonal	Rabbit	1:5,000	custom generated by COVANCE
Anti-Sis1	Rabbit	1:5,000	kind gift from Daniel Masison laboratory at NIDDK NIH
Anti-HA tag polyclonal	Rabbit	1:1,000	Abcam ab 184643
Anti-Pgk1 monoclonal	Mouse	1:5,000	Abcam ab 113687
Donkey anti-rabbit IgG-AP	Donkey	1:5,000	Santa Cruz Biotechnology sc-2057 used in Figure 2
Anti-rabbit IgG(Fc), AP Conjugate		1:7,500	Promega AP conjugate S3738 used in Figure 3

Anti-Btn2 and anti-Cur1 antibodies were generated by COVANCE using affinity purified (Ni-NTA agarose, Qiagen) proteins Btn2-His_6 and Cur1-His_6 produced from pET28-BTN2-HIS and pET28-CUR1-HIS.×

5 min incubation at 100°C and 5 min centrifugation, samples were loaded on precasted 4%–12% NuPAGE Bis-Tris gels (ThermoFischer Scientific) and electrophoresed for 35 min at 200 V in 1X NuPAGe MES SDS running buffer (ThermoFischer Scientific). Western blotting was performed on isopropanol activated PVDF membrane for 1 h 30 min in 1x NuPAGE transfer buffer (ThermoFischer Scientific) at 30 V. For detection of Btn2p, Sis1p and HA tag PVDF membranes were stained using SNAP i.d. 2.0 (Millipore) according to the recommendations of the manufacturer with 1X TBS (20 mM Tris Cl pH 7.4, 136 mM NaCl) with 0.1% Tween 20 as washing solution and 1X TBS, 0.1% Tween 20, 1% BSA as blocking solution (see Table 3 for antibodies). Before addition of secondary antibodies, membranes were washed once with blocking solution.

Construction of mutant strains

Disruption cassettes carrying the *kanMX* cassette were obtained by amplifying yeast genomic DNA of corresponding strains from the *S. cerevisiae* knockout collection (Winzeler *et al.* 1999) using a forward primer specific to the region 200 base pairs upstream of the start codon of the gene to be knocked out and a reverse primer specific to the region 200 base pairs downstream of the stop codon. Disrupted mutants were then obtained by transforming the resulting PCR fragment into yeast and selecting for G418-resistant colonies at a final concentration of 1 g/l of G418. In each case, disruption was confirmed by PCR.

Curing of [URE3] by overexpressed Btn2p or Cur1p

Strains carrying [URE3-1] were transformed with pBEE34 (*CUP1* promoter-*BTN2*) or pBEE42 (*CUP1* promoter-*CUR1*), and transformants were grown in 2 ml liquid SD media without adenine or tryptophan to stationary phase (OD_{600} ~3–5). The OD_{600} of the liquid cultures was measured and cells were transferred to 2 ml of dex -Trp liquid media containing 0.004% adenine and 0.250 mM copper sulfate starting at OD_{600} = 0.001. Cells were grown at 250 rpm in 14 ml tubes until they reached stationary phase, and dilutions were plated on ½ YPD plates. The percentage of red colored colonies was used as a measurement of [URE3] curing efficiency by Btn2 or Cur1 overproduction.

Hermes transposon mutagenesis to select Btn2-incurable mutants

The [URE3] strain BY241 [URE3] was transformed with pBEE34 (*CUP1*promoter-*BTN2*) and pSG36 [HERMES transposon with *natMX4* producing resistance to nourseothricin and *GAL1*- promoted HERMES transposase (Gangadharan *et al.* 2010)]. Cells were then grown for 2 days in filter-sterilized gal/raf liquid media (-Trp) starting from OD_{600} = 0.1. The transposase excises the HERMES transposon and inserts it into the genome (after which the plasmid is not functional). Cells were collected by centrifugation and placed in the same volume of the same liquid media containing additionally 1 g/L of 5-FOA and 0.004% uracil (5-FOA selects cells which have lost the *URA3*-carrying pSG36) and were grown overnight. Cells were collected by centrifugation and placed in the same medium containing additionally 0.1 g/L of nourseothricin sulfate (GoldBio) and grown overnight at 30°C. The culture was then diluted 300-fold in liquid media containing 6.67 g/L of yeast nitrogen base, 3% glucose, 1.92 g/L of SC-W drop-out mix, 0.01% adenine, 0.002% uracil, 1 g/L 5-FOA, 0.1 g/L of nourseothricin sulfate and 0.25 mM copper sulfate and cells were grown for two days to induce Btn2 overexpression. Finally, cells were spread on 1/2YPD plates. Clones which were not cured of the prion (still white) were selected for further analysis. The nat-resistant mutants were subjected to repeated copper induction of Btn2 to verify that [URE3-1] was not cured by Btn2p overproduction. Genomic DNA was purified (YeaStar Genomic DNA Kit, Zymo Research) from clones which showed Btn2-incurability but were curable by growth on 5 mM guanidine. The genomic DNA was digested overnight with *MseI*, cutting a site inside of the Hermes transposon and in the genomic DNA, and then treated with T4 DNA ligase overnight at 4°C. The region of genomic DNA containing part of the self-ligated *MseI*-digested Hermes transposon and the adjacent yeast genomic DNA was PCR amplified using primers BEE3054 (5'AGCACAATTGTACTCATAAG3') and BEE3055 (5'TCATTGATTCATCGACACTC3'): 4 ul of ligase mixture was used as a template for a 25 ul PCR reaction. PCR products were purified by QIAquick PCR purification kit (Qiagen) and were used as templates for a second round of PCR with primers BEE3049 (5'GCAAGTGGCGCATAAGTATCAAAATAAGCCACTTGTTG3') and BEE3056 (5'GTTTTGTCGTGTCGTTCTGCG3'. The resulting PCR products were purified by QIAquick PCR purification kit and sequenced using primers BEE3049 and BEE3056. If transposon insertions in the same gene were identified two or more times, this gene was knocked out in strain BY241 carrying [URE3-1] using the KanMX cassette from the knockout collection (G418-resistance). Btn2 overexpression curability of [URE3-1] was then tested in the newly generated mutant.

[URE3] seed number measurements

Cells were grown to single colonies on YPAD plates with 3 mM or 5 mM guanidine HCl. Single colonies were picked up in their entirety, suspended in 200 μL of water and each spread on an SC—Ade plate. The number of Ade+ colonies was counted and used as a representation of the prion seed number in the founding cell

of the colony (Cox *et al.* 2003). Ten colonies were examined for each strain in each experiment.

Cytoduction

Cytoplasmic transfer (cytoduction) was performed using the *kar1-1* mutation (Conde and Fink 1976) that largely blocks nuclear fusion after mating. Heterokaryons are formed by mating which, on cell division, produce haploids with the parental nuclei and a mixture of cytoplasms of the parents.

Microscopy

A Zeiss LSM 780 confocal scanhead (Carl Zeiss Microscopy, LLC, Thornwood, NY) mounted on a Zeiss AxioObserver.Z1 microscope, running ZEN 2.3 software, was used to collect 2 D images of red/green fluorescence and DIC images. The objective lens used was the Zeiss Plan-Apo 100x/1.4 Oil M27. For Ure2N-GFP fluorescence: excited by the 488 nm laser, emission was filtered by spectral detector band pass 420–475 and 500–610 nm. For Btn2-RFP fluorescence: excited by the 555 nm laser; emission was filtered by spectral detector long pass 560 nm. Differential Interference Contrast (DIC) images were acquired simultaneously with the Ure2N-GFP channel.

Preparation of samples for SILAC

Media (for 1 liter): Tyr 50 mg, Ade 80 mg, Asp 80 mg, His 20 mg, Ile 50 mg, Leu 100 mg, Met 20 mg, Phe 50 mg, Thr 100 mg, Trp 50 mg, Ura 20 mg, Val 140 mg, Pro 200 mg, YNB 6.7 g, and Glucose 20 g. For preparation of unlabeled media unlabeled L-Arg (20 mg/L) and L-Lys (30 mg/L) were added. For preparation of labeled media 20 mg/L of L-Arg-2HCl ($^{13}C_6$ 99%; $^{15}N_4$ 99%) and 30 mg/L of L-Lys-2HCl ($^{13}C_6$ 99%; $^{15}N_2$ 99%) were added. A pre-culture of cells carrying [URE3] and originating from a single colony (strains BEE1386, BEE1387, BEE1392, BEE1395, and BEE1401) was grown to stationary phase in 2 ml of 1X Synthetic Complete—Ade (Sunrise Inc.) liquid media with 0.67% YNB (Difco) and 2% glucose in a 14 ml cultivation tube. 12 µl of the pre-culture was inoculated in 60 ml of unlabeled or labeled media in a 250 ml flask and was grown to OD_{600} in the range 0.6–0.9. Then 50 ml of culture was placed into a 50 ml conical tube and spun for 3 min in a cold room (3000 g) and the pellet was transferred with cold water to a 1.5 ml microfuge tube. The tube was spun for 20 s in microcentrifuge at 4°C, supernatant was removed and the pellet was washed 3 times with cold water. The pellet was resuspended in 500 ul of cold water, frozen in ethanol with dry ice and placed at –80°C for storage until all samples were collected. Unlabeled wild-type samples were then mixed with equal amounts of labeled mutant samples (based on cell pellet weight). All procedures were made on ice. There were three repeats of each mix of samples. Mixed samples were pelleted in a microcentrifuge, the supernatant was removed and cells were resuspended in 600 ul of lysis buffer (1% sodium dodecanoate, 8 M urea, 1 mM dithiothreitol, 1 mM EDTA, 100 mM PMSF, 1x complete EDTA free protein inhibitor, 5% glycerol, 1 M Tris-HCl pH 7.4) in a 2 ml tube with screw cap. Glass beads were added almost to the top of the tube and cells were homogenized in the cold room for 3 minutes. Using a hot needle, a hole was poked in the bottom of the 2 ml tube, it was placed into a 5 ml tube and subjected to centrifugation at 4000 g for 5 min at 4 C. The supernatant was placed in a 1.5 ml microfuge tube, centrifuged for 5 min at 4 C in a microcentrifuge and the supernatant was placed to a new microfuge tube. An aliquot of the supernatant was taken for BCA assay and the rest of the supernatant was stored at −80°C before trypsinolysis, purification and mass spectrometry. After further reduction and alkylation of thiols samples were digested with trypsin, subjected to off-line high pH concatenated separation, and subjected to LC/MS/MS with data analysis using MaxQuant (Rappsilber *et al.* 2007; Cox and Mann 2008; Wang *et al.* 2011) (details in Edskes *et al.*, submitted).

Statistics

The curing data follows the binomial distribution, because each data point expresses two alternative results, loss of [URE3] or its retention. The number of observations (N), the probability of curing (p) and of not curing (q) are used in calculations. The results should be approximately normally distributed as long as $Npq \gg 1$, which was true for all our data. We want to calculate the probability that two sets of data (*e.g.*, w.t. *vs* mutant) could be samples from the same population. If pw, qw, Nw *vs* pm, qm, Nm are the parameters for w.t. and mutant, respectively, then, combining populations, the overall chance of prion loss is $p = (pw \bullet Nw + pm \bullet Nm)/(Nw+Nm)$, expected loss number in the mutant clones is $p \bullet Nm$ with standard deviation $S = ((Nw+Nm)p(1-p))^{1/2}$. The difference $p \bullet Nm - pm \bullet Nm$ between expected and observed number of mutant clones that lost the prion, is divided by the standard deviation S, and the probability of this difference or greater from the expected result is obtained from a normal distribution table. Data in Supplementary Figure S2 was analyzed using the Mann-Whitney *U*-test.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, tables, and Supplementary material which are available at figshare: https://doi.org/10.25386/genetics.13549292.

Results

Isolation of mutants with impaired Btn2p or Cur1p curing of [URE3-1]

In order to find which genes are involved in the process of Btn2p overproduction curing of [URE3], strain BY241 was transformed with pBEE34 carrying *BTN2* under the *CUP1* promoter and by pSG36 carrying a HERMES transposon containing *NatMX* (nourseothricin-resistance) and its transposase under the *GAL1* promoter. Transposition was induced in galactose, then cells lacking the transposon-carrying plasmid were selected. Among these cells, those resistant to nourseothricin, and thus presumably carrying an insertion of HERMES in the genome, were selected. Then Btn2p overproduction was induced by adding copper (Supplementary Figure S1) and, after a period of growth under this condition, cells were plated on ½ YPD to screen for those retaining [URE3] by their white colony color (Ade+). [URE3] cells have much reduced Ure2p repressor activity, consequently increased *DAL5*-promoted *ADE2* activity and are Ade+ (white). [ure-o] cells have normal Ure2p repression of the *DAL5* promoter, little transcription of the *DAL5: ADE2* reporter, and accumulation of an adenine pathway intermediate that forms a red pigment. DNA was isolated from these mutants resistant to Btn2-curing of [URE3] and the site of the HERMES insertion was identified by PCR. Their resistance to curing was confirmed by repeated Btn2p overproduction in copper-containing media, and their sensitivity to guanidine-curing [by inhibition of Hsp104, needed for prion propagation (Ferreira *et al.* 2001; Jung and Masison 2001; Jung *et al.* 2002)] confirmed that the Ade+ phenotype was still due to the [URE3] prion. Strains with insertions in *UBR2* and *RPL4A* and upstream of *RPL21B* showed greatly

Table 4. Btn2p and Cur1p overproduction (under the *CUP1*-promoter) curing of [URE3] in mutants and wild-type

Host	Overproduction			
	Btn2p		Cur1p	
	#	% Curing	#	% Curing
WT	732	60	209	90
hsp42Δ	614	0.5*	112	12*
ubr2Δ	708	1.6*	112	9*
ubr2Δ rpn4Δ	354	59††	110	21
rpl21bΔ	1152	1.1*	122	8*
rpl4aΔ	1063	0.7*	150	46*
WT	—	—	99	29
ubr2Δ	—	—	85	3*
ubr2Δ rpn4Δ	—	—	84	31**
WT	361	94	290	76
rpl21bΔ	251	7*	272	6*
rpn4Δ#1	580	57	393	54
rpn4Δ#2	207	70	423	65
rpl21bΔ rpn4Δ	246	67†	162	55†

Cells were grown with inducing concentrations of copper for 12 generations before plating. The stability of [URE3] in the wild-type and mutant strains was tested before introduction of the CUR1/BTN2 overexpression plasmids as well as a 0 mM copper control for the WT (using "copper-free" YNB). Loss of [URE3] was <1% in all of those cases.
* = differs from w.t. with $p < 2 \times 10^{-4}$,
** = differs from *ubr2Δ* with $p < 2 \times 10^{-3}$,
† = differs from *rpl21bΔ* with $p < 10^{-4}$,
†† = differs from *ubr2Δ* with $p < 10^{-4}$.

reduced curing of [URE3] by overproduced Btn2. Replacing the Btn2p over-expression plasmid did not improve the curing ruling out a plasmid mutation as the basis of the lack of curing. To confirm that the transposon insertion in these strains was producing the reduction in curing, we made knockouts in the corresponding genes. These knockout strains showed almost no loss of [URE3] on overproduction of Btn2p (Table 4, Figure 1). *RPL4A* and *RPL21B* are duplicated genes encoding 60S ribosomal proteins, and each is the major source of the respective protein, with comparable slowing of growth and depletion of 60S subunits in *rpl4aΔ* and *rpl21b* strains (Steffen *et al.* 2008; Pillet *et al.* 2015). Ubr2p is an ubiquitin ligase that has as one of its targets Rpn4, a transcription factor stimulating expression of proteasomal genes (Wang *et al.* 2004).

Subclones of the deletion mutants that had lost the Btn2 overproduction plasmid were obtained and pBEE42 carrying *CUP1*-promoted *CUR1* was introduced. Both *ubr2Δ* and *rpl21bΔ* strains were resistant to curing by Cur1 overproduction (Table 4, Supplementary Figure S2).

Has an incurable [URE3] variant been selected?

Curability by normal levels of Btn2p and Cur1p was often an unstable property of newly isolated [URE3] variants, with relatively curing-resistant derivatives arising frequently even in a *btn2Δ cur1Δ* host (Wickner *et al.* 2014). [URE3] from Btn2p-incurable mutants *hsp42Δ*, *ubr2Δ*, *rpl4aΔ*, *rpl21bΔ* or 1021 (wild-type) was cytoduced into wild-type recipient 4591, five cytoductants in

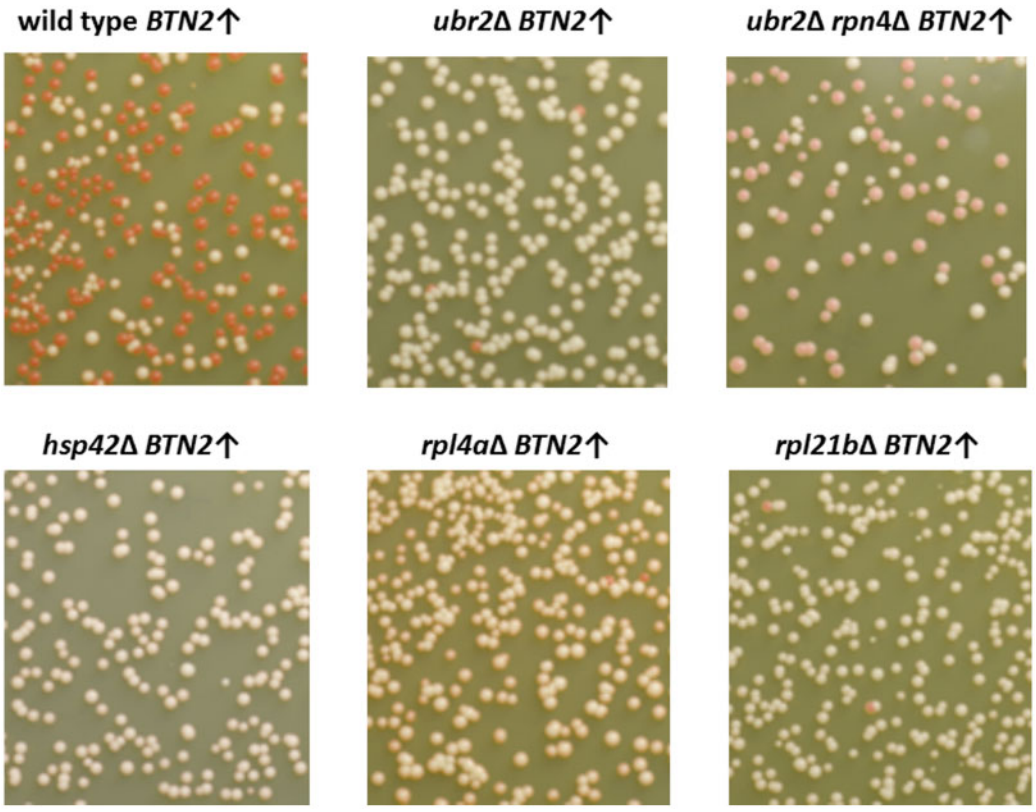

Figure 1 Loss of Btn2-overproduction curing of [URE3] in mutants. [URE3-1] strains carrying pBEE34 (*CUP1* promoter—*BTN2*) were inoculated in synthetic complete minus tryptophan media containing 0.25 mM copper sulfate, starting at $OD_{600} = 0.001$, grown for 12 generations and dilutions were plated on ½ YPD to score loss of [URE3]. Red color development by *ubr2Δ rpn4Δ* cells was less than other strains and the Ade- phenotype and loss of [URE3] was confirmed by testing on synthetic complete−Ade plates and mating with [ure-o] cells (Supplementary Figure S7). The stability of [URE3] in the wild-type and mutant strains was tested before introduction of the *CUR1*/*BTN2* overexpression plasmids and loss of [URE3] was <1% in all of those cases.

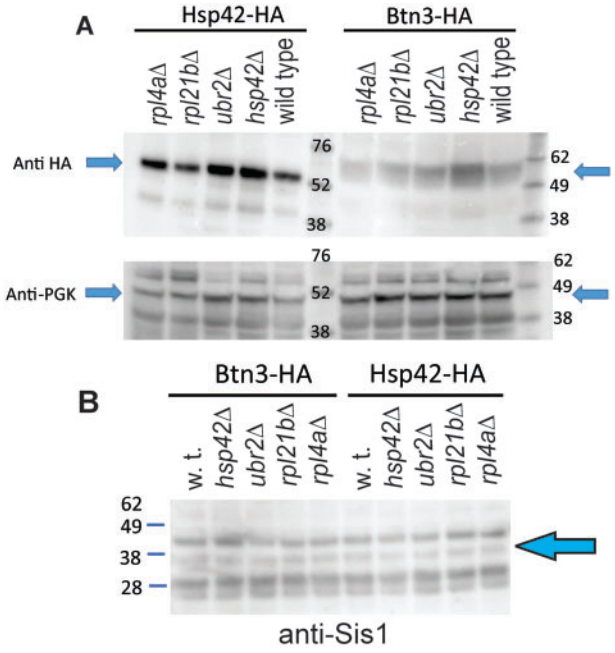

Figure 2 Western blot analysis of Hsp42, Btn3 and Sis1 levels in incurable mutants. (A) Hsp42-HA and Btn3-HA were expressed from their own promoters on CEN plasmids in log phase cells and were visualized by staining with anti-HA tag antibodies. Anti PGK staining was used as a loading control. Btn3 is 57.6 kDa; Hsp42 42.8 kDa; PGK1 44.7 kDa, and the HA tag adds 1.1 kDa. For Western blots 10 μg of protein per lane was loaded. (B) Anti-Sis1 staining of lysates of wild-type and Btn2-incurable mutants. Strains carried CEN plasmids expressing Btn2-HA and Hsp42-HA from their own promoters.

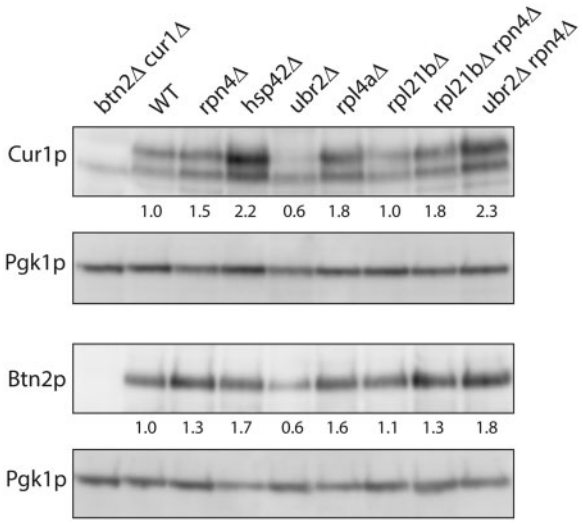

Figure 3 Btn2p and Cur1p levels in incurable mutants. After pregrowth on 3 mM guanidine HCl to remove [URE3], cells were grown in SC-Trp with 250 μM copper sulfate. Extracts (10 μg per lane) were loaded on a 12% MOPS polyacrylamide gel. The primary antibody was anti-Btn2p or affinity-purified rabbit anti-Cur1p, and the secondary antibody was Promega anti-rabbit IgG(Fc) AP conjugate S3738. Cur1p has a predicted molecular mass of 29.2 kDal and Btn2p is 47.1 kDa. Lane 1: BEE1012 *btn2Δ cur1Δ*; Lane 2: BY241 wild-type; Lane 3: BEE1319 *rpn4Δ*; Lane 4: BEE1190 *hsp42Δ*; Lane 5: BE1192 *ubr2Δ*; Lane 6: BEE1272 *rpl4aΔ*; Lane 7: BEE1251 *rpl21bΔ*; Lane 8: BEE1417 *rpl21bΔ rpn4Δ*; Lane 9: BEE1318 *ubr2Δ rpn4Δ*.

each case were transformed by a plasmid with *BTN2* or *CUR1* under the *CUP1* promoter and transformants were subjected to copper induction. All cytoductants analyzed were curable upon Btn2p/Cur1p overproduction (Table 5, Supplementary Figure S3), indicating that the [URE3-1] variant used had not changed to an incurable form. Other attempts to select a variant of [URE3] incurable by Btn2p- or Cur1p- overproduction were unsuccessful.

Western blot analysis of lysates of Btn2p-incurable mutants

Lower levels of Btnp2 or Cur1p overproduction (Kryndushkin *et al.* 2008), overproduction of Sis1p (Malinovska *et al.* 2012; Wickner *et al.* 2014), decreased Hsp42 (Wickner *et al.* 2014) or elevated Btn3 (Kanneganti *et al.* 2011) could explain the failure of [URE3] curing in these mutants. We examined levels of Btn2p, Cur1p, Sis1p, Hsp42-HA, and Btn3-HA in mutants and wild-type lysates by western blot (Figures 2 and 3). Levels of Sis1p and Hsp42-HA did not change in the Btn2-incurable mutants. The level of overproduced Btn2p was lower in the *ubr2Δ* mutant than in wild-type, but *rpl4aΔ* and *rpl21bΔ* did not show this effect (Figure 3, Supplementary Figure S4). Levels of Btn3-HA did not increase in the mutants, except in the *hsp42Δ* mutant (Figure 2B). Because elevated Btn3p prevents curing of [URE3] by elevated Btn2p (Kanneganti *et al.* 2011), it is possible that our observed increase in Btn3p in the *hsp42Δ* mutant is part of the explanation for failure of [URE3] curing by Btn2p overproduction in this strain (Wickner *et al.* 2014).

The levels of overproduced Cur1p were lower in the *ubr2Δ* strain, explaining the incurability of [URE3] in this strain. Cur1p is substantially higher in the *hsp42Δ* host (Figure 3, Supplementary Figure S4). Cur1p was not lower in the *rpl4aΔ* or *rpl21bΔ* strains, although both strains were resistant to curing by overproduced Cur1p (Table 4). The reduced levels of Cur1p in *ubr2Δ* strains are restored to above-normal levels by including the *rpn4Δ* mutation that prevents overactivity of the proteasome system (Figure 3, Supplementary Figure S4).

Table 5 Cytoduction of [URE3] from Btn2-incurable mutants into wild-type recipient and subsequent Btn2p or Cur1p overproduction

Cytoductants into WT from	% cured upon Btn2↑	% cured upon Cur1↑
Wild-type	28–65	15–61
hsp42Δ	24–50	27–53
ubr2Δ	44–58	20–44
rpl21bΔ	8–62	22–49
rpl4aΔ	32–60	43–65

To test whether the [URE3] prion in incurable mutants was the incurable property, cytoplasm was transferred from the curable w.t. (BY241 [URE3-1]) or the isogenic incurable mutants to the w.t. [ure-o] strain 4,591. Five cytoductants for each case were transformed with pBEE34 (*CUP1-BTN2*) or pBEE42 (*CUP1-CUR1*), grown 13–14 generations with Cu and plated on ½ YPD. The stability of [URE3] in these strains was tested before introduction of the *CUR1/BTN2* overexpression plasmids and loss of [URE3] was <1% in each cases. Dot plot of data is shown in Supplementary Figure S3. The Mann-Whitney *U*-test shows there is no statistical difference between the wild-type and any of the mutants.

Colocalization of Btn2p with Ure2p in mutants

Btn2-RFP and Ure2N-GFP were expressed from different plasmids in wild-type and Btn2-incurable mutants. Living cells then were subjected to microscopy. It was found that Btn2-RFP foci still largely colocalized with Ure2N-GFP aggregates in *ubr2Δ*, *rpl4aΔ*, and *rpl21bΔ* as well as in wild-type, but did not colocalize in *hsp42Δ* (Figure 4, Supplementary Table S1).

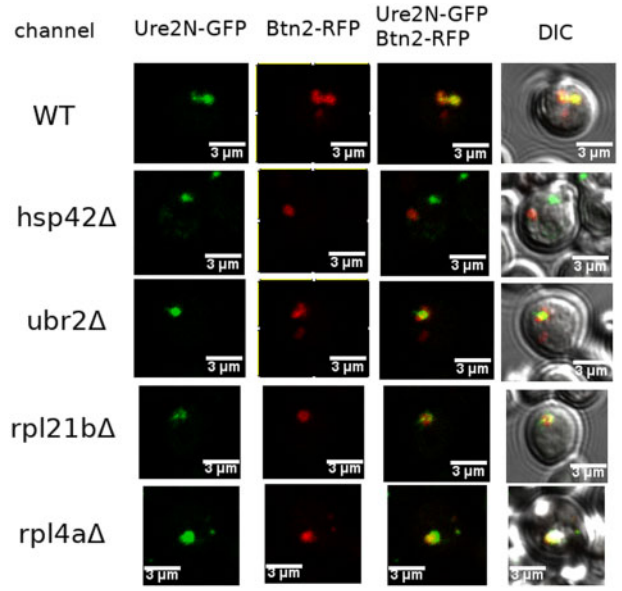

Figure 4 Co-localization of Btn2-RFP and Ure2-GFP in wild-type, *ubr2*Δ, *rpl4a*Δ, and *rpl21b*Δ, but not in *hsp42*Δ incurable mutants. Btn2-RFP was expressed from pBEE28 under a *GAL1* promoter, and Ure2N-GFP was expressed from pBEE29 under a constitutive promoter. Wild-type and mutant cells were transformed by both pBEE28 and pBEE29 plasmids. Cells grown to stationary phase in liquid synthetic media with 3% raffinose were diluted to $OD_{600} = 0.5$ in liquid synthetic media with 2% raffinose and 3% galactose, were grown overnight and then subjected to microscopy.

Seed number of [URE3] in Btn2p-incurable mutants

At normal levels, Btn2p and Cur1p cure [URE3] prion variants with particularly low seed numbers, but not those with higher seed numbers, which can only be cured by overproducing the curing proteins (Wickner *et al.* 2014). Seed number of [URE3-1] in *ubr2*Δ, *rpl4a*Δ, and *rpl21b*Δ was determined using the guanidine method of Cox *et al.* (2003) (see Material and Methods section) and showed no consistent increase in these mutants in comparison with wild-type in three experiments (Figure 5). Paradoxically, *hsp42*Δ strains showed a modest tendency toward lower seed numbers than wild-type, even though they were resistant to curing (Figure 5).

Btn2p or Cur1p overproduction curing of [URE3] in Hsp104 T160M mutants

Overproduction of Hsp104 is best known for its curing of [PSI+] (Chernoff *et al.* 1995), but overproduced Hsp104 can also, less efficiently, cure [URE3] (Kryndushkin *et al.* 2008; Matveenko *et al.* 2018). Mutants in the N-terminal domain of Hsp104 eliminate its curing ability while not affecting its ability to promote prion propagation (Hung and Masison 2006). We overproduced Btn2p or Cur1p in one such mutant, Hsp104 T160M, and found that curing proceeded efficiently, indicating that the Hsp104 curing activity is not required for the curing of [URE3] by Btn2p or Cur1p overproduction (Table 6, Supplementary Figure S5). In addition, co-overproduction of Hsp104 with Btn2p did not affect curing of [URE3].

Rpl4Ap overproduction does not cure [URE3]

As deletion of *RPL4A* stabilizes [URE3] against challenge by Btn2p or Cur1p, it was possible that Rpl4Ap has an anti-prion action

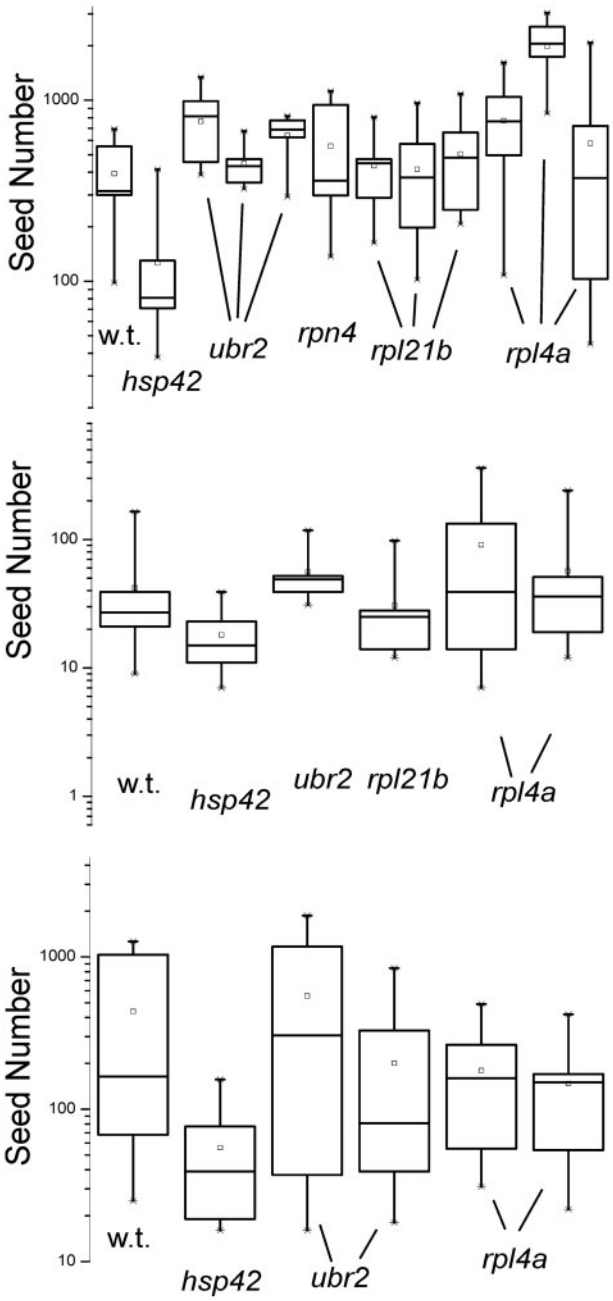

Figure 5 Propagon/seed number of [URE3-1] in wild-type and Btn2-incurable mutants. Strains with [URE3-1] were plated on YPAD containing guanidine (3 mM in the top and bottom panels, 5 mM in the middle panel) to inhibit Hsp104 (Jung *et al.* 2002) and thus block prion propagation by preventing filament cleavage (Ness *et al.* 2002). Each of 10 colonies was resuspended in water and plated on SC-Ade, and the number of colonies forming is a measure of propagon number (Cox *et al.* 2003). Separate experiments with the same mutant are shown as separate "whisker" diagrams.

independent of Btn2p/Cur1p overproduction. However, we find that *GAL1*—promoted Rpl4Ap overproduction does not cure the [URE3-1] prion in strain BY241 (Supplementary Figure S6). We also examined 96 [URE3] variants generated in an *rpl4a*Δ host, and find that none were destabilized on restoration of *RPL4A* by mating with a wild-type host. Thus, there are no (or very few) [URE3] variants for which Rpl4Ap by itself has an anti-prion action.

Table 6 Btn2 or Cur1 overproduction curing of [URE3] in Hsp104 T160M mutants

Strain	Prion curing (%)
WT BTN2↑	94–98
T160M BTN2↑	83–94
WT CUR1↑	77–95
T160M CUR1↑	52–83

Wild-type (strains BEE1201, BEE1203) and *hsp104(T160M)* mutants (strains BEE1200 and BEE1202) carrying [URE3-1] were transformed with pBEE34 (*CUP1-BTN2*) or pBEE42 (*CUP1-CUR1*), grown with $CuSO_4$ to induce Btn2/Cur1 production and plated on ½ YPD to measure curing. A total of >1,500 colonies were counted in each case and the range of curing frequency is shown. The stability of [URE3] in the wild-type and mutant strains was tested before introduction of the *CUR1/BTN2* overexpression plasmids as well as a 0 mM copper control for the WT (using "copper-free" YNB). Loss of [URE3] was <1% in all of these cases. See also Supplementary Figure S5.

Deficiency of 60S ribosomal subunit genes prevents Btn2/Cur1 overproduction curing of [URE3]

To determine whether the effects on prion curing of *rpl21b* is paralog—specific, we tested an *rpl21aΔ* mutant, and found that it too prevented [URE3] curing by overproduced Btn2p or Cur1p (Table 7). Both *rpl21aΔ* and *rpl21bΔ* noticeably slow cell growth (Steffen *et al.* 2008; Bhattacharya *et al.* 2010; Yoshikawa *et al.* 2011) indicating that each contributes substantially to the pool of the Rpl21 ribosomal protein. Indeed, we find that Btn2p and Cur1p overproduction curing of [URE3] was inhibited in *rpl11bΔ* and *rpl16bΔ* mutants which decrease 60S ribosomal subunits levels, but curing was still occurring in *rps14aΔ* and *rps30bΔ* strains which decrease 40S ribosomal subunits levels (Figure 6). Thus, 60S ribosomal subunit deficiency, rather than slowing of translation per se, seems to be the factor preventing prion curing. Note that *rps14aΔ* and *rps30bΔ* each limit translation enough to slow growth as much as *rpl21bΔ* or *rpl11bΔ* (Steffen *et al.* 2008; Bhattacharya *et al.* 2010; Yoshikawa *et al.* 2011).

Restoration of Btn2/Cur1 overproduction curing of [URE3] in *ubr2Δ rpn4Δ* and *rpl21bΔ rpn4Δ* double mutants

Ubr2 is an E3 ubiquitin ligase whose target is Rpn4, a transcription factor promoting expression of proteasome components (Wang *et al.* 2004). If the abrogation of Btn2/Cur1-curing of [URE3] is because of this action of Ubr2p, we expected that in a *ubr2Δ rpn4Δ* double mutant Btn2p or Cur1p overproduction and prion curing would be restored in comparison with a single *ubr2Δ* mutant. Indeed this is the case (Table 4, Figures 1 and 3). The *ubr2Δ rpn4Δ* double mutant colonies cured of [URE3] were pink on ½ YPD (instead of red as in wild-type), but were Ade- (Supplementary Figure S7A), and curing was confirmed by mating of the pink mutant colonies with wild-type [ure-0] cells and replica plating them on a media without adenine (Supplementary Figure S7B). Moreover, the reduced levels of Cur1p found in *ubr2Δ* strains were restored in the *ubr2Δ rpn4Δ* double mutant (Figure 3).

Because mutations in 60S ribosomal subunit proteins (but not in 40S proteins) result in elevated proteasome components (Cheng *et al.* 2019), we examined whether *rpn4Δ* would reverse the effects of *rpl21bΔ* mutations on curing of [URE3] by Btn2p or Cur1p. Indeed, we found that in a *rpn4Δ rpl21bΔ* strain, either Btn2p or Cur1p cured [URE3] with efficiencies similar to what is found in a wild-type host (Table 4).

Table 7 Btn2 and Cur1 overproduction curing of [URE3] prion is impaired in *rpl21aΔ* mutant (*RPL21A* is a paralog of *RPL21B*)

Sample plated on ½ YPD	Total	% Curing
Wild-type Btn2↑	612	71
***rpl21aΔ*#1 Btn2↑**	580	3*
***rpl21aΔ*#2 Btn2↑**	460	2*
Wild-type Cur1↑	846	41
***rpl21aΔ*#1 Cur1↑**	564	4*
***rpl21aΔ*#2 Cur1↑**	592	4*

Wild-type strain BEE1021 and two verified *rpl21aΔ* knockout isolates (#1, #2) were transformed with pBEE34 (*CUP1-BTN2*) or pBEE42 (*CUP1-CUR1*) and Btn2p or Cur1p overproduction was induced by growth with $CuSO_4$ as described in Methods. Aliquots of the indicated dilutions of each culture were plated on ½ YPD and red/white colonies were scored. The stability of [URE3] in the wild-type and mutant strains was tested before introduction of the *CUR1/BTN2* overexpression plasmids as well as a 0 mM copper control for the WT (using "copper-free" YNB). Loss of [URE3] was <1% in all of these cases.
* = differs from w.t. with $p < 10^{-4}$.

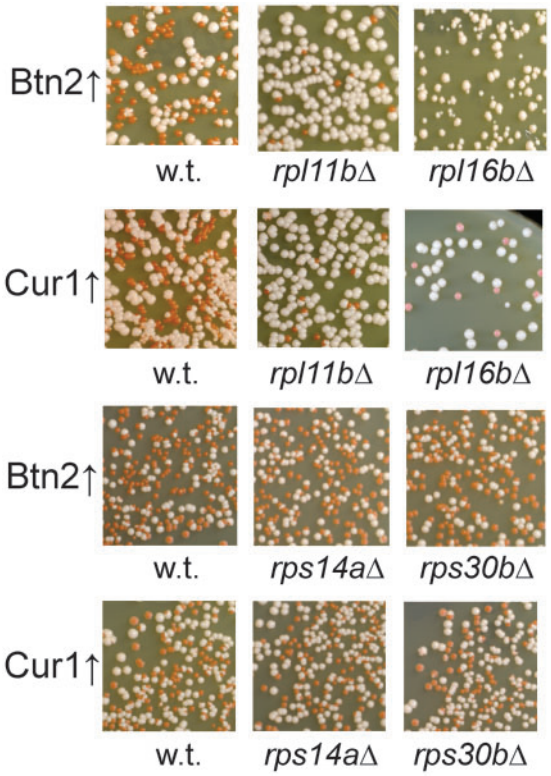

Figure 6 60S ribosomal subunit deficiency prevents [URE3-1] curing. Mutants in genes encoding 60S ribosomal subunit proteins carrying [URE3-1] and overproducing Btn2p or Cur1p were examined as described in section Methods. The stability of [URE3] in the wild-type and mutant strains was tested before introduction of the *CUR1/BTN2* overexpression plasmids and loss of [URE3] was <1% in all cases.

Btn2p overproduction curing of [URE3] in mutants of Btn2p-interactors

In its protein—trafficking role, Btn2p interacts with SNARE and endosome proteins (Kama *et al.* 2007). Btn2p overproduction was performed in knockouts of genes shown to interact with Btn2p (Kama *et al.* 2007) but there was little influence on Btn2p overproduction curing in these mutants in comparison with wild-type (Table 8).

Table 8 Btn2 overproduction (from copper promoter) curing in mutants of genes shown to interact with Btn2 (Kama *et al.* 2007)

Strain	Btn2 overproduction		Cur1 overproduction	
	Colonies	% Curing	Colonies	% Curing
Wild-type	637	69	408	59
snc1Δ	538	68	349	70
snc2Δ	417	87	230	73
pep8Δ	301	88	233	81
vps27Δ	401	79	559	66
tlg2Δ	224	34	248	50
vps35Δ	419	92	433	84

Strain BY241 (w.t.) and isogenic knockout strains were tested for curing as described in Methods. The stability of [URE3] in the wild-type and mutant strains was tested before introduction of the *CUR1/BTN2* overexpression plasmids as well as a 0 mM copper control for the WT (using "copper-free" YNB). Loss of [URE3] was <1% in all of these cases. In all mutants, robust curing was observed by overproduction of either Btn2 or Cur1. While there were statistically significant differences (*e.g.*, the *tlg2Δ* strain was slightly more slowly for curing by Btn2p, while the *pep8 Δ* and *vps35Δ* were slightly more rapidly cured by Btn2 or Cur1, *vps27Δ* by Cur1 and *snc2Δ* by Btn2), all were well cured.

Generation of [PSI+] variants curable by Cur1p overproduction

None of the three [PSI+] variants previously tested were cured by Btn2p or Cur1p overexpression (Kryndushkin *et al.* 2008; Barbitoff *et al.* 2017). We examined five different [PSI+] variants (strong [PSI+], Sc4, Sc37, Lieb W, and Lieb S), transforming each with 2 μ plasmids carrying BTN2 (p1289) or CUR1 (p1488) under their own promoters and no curing was observed.

In strain 74-D694 carrying *BTN2* (pBEE1) or *CUR1* (pBEE2) under a *GAL1* promoter on a CEN plasmid and pSL1066 with *CUP1*-promoted *SUP35-NM-GFP*, we generated [PSI+] variants by growth with $CuSO_4$. Sixty *BTN2*- and 60 CUR1- plasmid containing [PSI+] isolates were streaked on minimal dextrose or galactose plates with limited adenine (10 mg/l). Nine clones for CUR1- and 4 clones for BTN2-overexpression showed more red or darker pink color on galactose than on dextrose plates. In the next step, candidates were streaked on YPAD plates to remove plasmids and retransformed with an empty vector or a plasmid with *BTN2* or *CUR1*. Three subclones of each Btn2/Cur1-sensitive [PSI+] variant were analyzed. The effect of curing was reproduced only for 1 subclone (out of 3 subclones) for 2 CUR1-sensitive [PSI+] clones (Figure 7). Thus, these variants are unusual and quite unstable.

SILAC of Btn2-incurable mutants

To detect proteins whose elevated or depressed levels may be affecting Btn2 curing, we measured the ratio of over 4600 proteins from isogenic mutant and wild-type cells using SILAC (see Methods section), labeling one with L-Arginine-$^{13}C_6$,$^{15}N_4$ hydrochloride and L-Lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride, and the other with ^{12}C, ^{14}N amino acids. Cells were mixed, extracted, and proteins were digested with trypsin, so that each peptide had one amino acid differently labeled between mutant and wild-type. Digested extract mixtures were analyzed by mass spectrometry (ONG *et al.* 2002).

Several proteins showed modest changes in curing-resistant strains compared to the isogenic wild-type (Supplementary Tables S2 and S3), some with evident connection to the auxotrophies of these strains. None of these seemed clearly related to the curing process. However, Tma10p was dramatically elevated in *hsp42Δ*, *rpl4aΔ*, and *rpl21bΔ* strains 28-, 22-, and 39-fold, respectively, but only slightly elevated in *rps30bΔ*, which does not affect

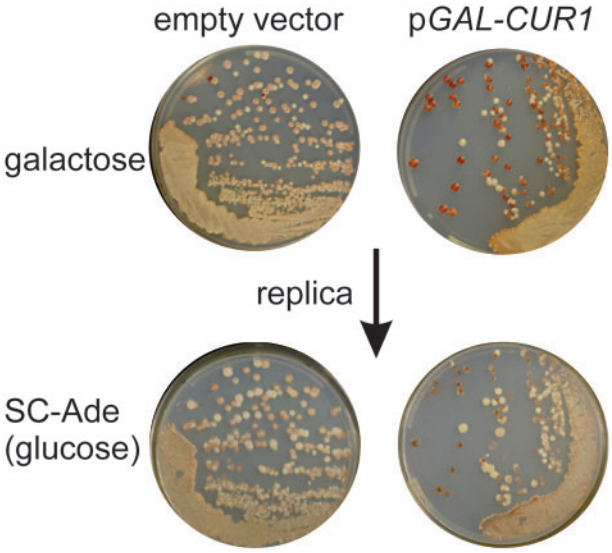

Figure 7 Rare [PSI+] variant sensitive to curing by Cur1p overproduction. In strain 74D-694 carrying pBEE2 (*CEN LEU2 GAL1*promoter-*CUR1*) and pSL1066 (*CEN URA3 CUP1*promoter-*SUP35NM-GFP*) [PSI+] variants were copper-induced. Cur1-sensitive colonies were found by subcloning on galactose media and picking those which became [psi-]. The original [PSI+] Cur1-sensitive variants were streaked on YPAD plates to allow loss of pBEE2 and pSL1066, were retransformed with pBEE2 or empty vector control (pH316) and tested for Cur1-sensitivity again by streaking cells on selective plates containing 2% galactose and 10 mg/l adenine. The galactose plates were replica plated to dextrose plates lacking adenine (below).

Btn2p curing of [URE3]. It was possible that elevated levels of Tma10p would antagonize Btn2p's curing activity, or that Tma10p is necessary for [URE3] propagation. Overexpressing Tma10p from a *GAL* promoter (p1678) in parallel with *GAL*-promoted Btn2p (pBEE1), did not, however, diminish the curing efficiency in a wild-type host (Supplementary Table S3). Likewise, a BY241 *tma10::kanMX* host (5991) maintained [URE3-1] as stably as an isogenic wild-type.

Changes in Hsp42, Hsp104, Sis1p, Ydj1p, Swa2p, Cpr7, Btn3p, or Ure2p itself could affect loss of the prion (Aigle and Lacroute 1975; Moriyama *et al.* 2000; Higurashi *et al.* 2008; Kanneganti *et al.* 2011; Wickner *et al.* 2014; Kumar *et al.* 2015; Troisi *et al.* 2015). None of these proteins were changed significantly more in *rpl4a* and *rpl21b* than in *rps30a* (Table 9).

Discussion

We used two approaches to find genes affecting Btn2p and Cur1p curing of the [URE3] prion. We examined, as candidates, genes known to be involved in Btn2p's action in protein sorting. Btn2 interacts in two-hybrid assays with Snc1 and Snc2, and binds in extracts to Tlg1, Tlg2 and Vti1, as well as Snc1 and Snc2, all endocytic SNARE components mediating membrane fusion of vesicles with endosomes (Kama *et al.* 2007). Btn2p also co-localizes with Vps27p and co-IPs with Vps26p and Vps35p. We find that *snc1Δ*, *snc2Δ*, *tlg2Δ*, *vps27Δ*, *vps26Δ*, and *vps35Δ* (*TLG1* and *VTI1* are essential) have no substantial effect on the Btn2p overproduction curing of [URE3], suggesting that Btn2p has two separate functions. Of course, Btn3p, shown to bind to Btn2p, inhibits both activities when overproduced (Kanneganti *et al.* 2011).

We also used HERMES transposon mutagenesis in an unbiased screen for genes whose mutation would interfere with curing of

Table 9 SILAC levels of some proteins affecting [URE3]

Mutant=> Protein	*rpl4aΔ*/w.t.	*rpl21bΔ*/w.t.	*rps30bΔ*/w.t.
Hsp42	1.34 ± 0.07	1.27 ± 0.27	1.27 ± 0.16
Btn2	NSD	NSD	NSD
Cur1	NSD	NSD	NSD
Hsp82	0.84 ± 0.25	0.82 ± 0.24	0.80 ± 0.37
Hsp104	0.95 ± 0.14	0.99 ± 0.22	0.94 ± 0.34
Rpn4	NSD	NSD	NSD
Ubr2	NSD	NSD	NSD
Ubi4	0.90 ± 0.02	0.93 ± 0.07	0.94 ± 0.13
Sis1	0.86 ± 0.06	0.86 ± 0.05	0.91 ± 0.04
Ydj1	0.74 ± 0.04	0.82 ± 0.06	0.83 ± 0.04
Hsp26	1.50 ± 1.1	1.50 ± 1.9	1.4 ± 1.9
Swa2	0.78 ± 0.03	0.86 ± 0.07	0.82 ± 0.2
Ure2	0.95 ± 0.11	0.99 ± 0.06	0.99 ± 0.25
Btn3	0.76 ± 0.15	0.81 ± 0.12	0.83 ± 0.17
Cpr7	0.86 ± 0.02	0.94 ± 0.05	0.90 ± 0.13

Each ratio was the result of three independent determinations (mean ± standard deviation). NSD = not sufficient data.

[URE3] by Btn2p or Cur1p. Our screen identified *rpl4a*, *rpl21b*, and *ubr2* mutants with this property. Deletion mutants of these genes had the same effect. Curing was also diminished by *rpl21a*, *rpl11b*, or *rpl16b*, but not by *rps14a* or *rps30b*, indicating that 60S subunit deficiency was specifically involved, but not a particular 60S ribosomal subunit protein.

Consequences of 60S subunit deficiency

Mutation of ribosomal protein genes has the potential to produce specific phenotypes by a variety of mechanisms. Fifty-nine ribosomal proteins are encoded by paralogous pairs of genes, which can differ in encoded sequence, but in the most carefully studied cases, differences in paralog expression levels have explained the observed phenotype differences (*e.g.*, Palumbo *et al.* 2017). The extensive modifications of rRNAs and ribosomal proteins could produce a large array of distinct ribosomes, but there is little evidence that this produces functional varieties (reviewed by Dinman 2016). Extraribosomal functions of ribosomal proteins have been documented in a few cases (Blumenthal and Carmichael 1979), reviewed in Warner and McIntosh (2009), but since all 60S ribosomal protein gene deletions tested suppressed curing, it is not likely an extraribosomal effect here. An overall deficiency of active ribosomes might be expected to retard translation of some mRNAs more than others, but should be produced equally by either 40S or 60S deficiency. However, several cases of defects selective for 60S subunits rather than 40S have been described.

Mutations in any of 30 chromosomal genes (*MAK* for maintenance of killer) lose the M_1 dsRNA satellite dsRNA of the L-A virus and have lowered copy number of L-A itself (Wickner 2013). Eighteen of the 30 *MAK* genes were deficient in 60S ribosomal subunits, many encoding 60S subunit proteins (Ohtake and Wickner 1995). None had 40S subunit protein defects. It has been suggested that impaired expression of the viral nonpolyA mRNA relative to cellular polyA+ mRNAs (Edskes *et al.* 1998) combined with the preferential use of the viral coat proteins by the L-A helper virus, can explain the preferential loss of the satellite segment. Except for viral mRNAs, there are no known nonpolyA mRNAs in *S. cerevisiae*.

60S subunit deficiency also prolongs the replicative life span of *S. cerevisiae*, the number of daughter cells that can be made by a single mother cell (Steffen *et al.* 2008). This phenomenon requires the action of Gcn4p (Steffen *et al.* 2008), best known for its role in the general control of amino acid biosynthesis (Hinnebusch 2005). Gcn4p expression requires the read-through of several small upstream open reading frames, and 60S subunit deficiency is known to facilitate this readthrough (Martín-Marcos *et al.* 2007). While *rpl21bΔ* showed life span extension, *rpl4aΔ*, *rpl11bΔ*, *rpl16bΔ*, and *rpl21aΔ* did not consistently do so (Steffen *et al.* 2008), suggesting that the prion curing suppression effect was not due to elevated Gcn4p. Moreover, our SILAC data did not show significant changes in Gcn4p. However, there are other yeast genes known to have small upstream ORFs which may be involved (Lawless *et al.* 2009).

Recently, Tye *et al.* showed that limiting 60S subunit biogenesis by lowering levels of certain rRNA processing RNAses, results in elevated Hsf1p, a transcription factor promoting heat shock protein and other proteostasis factor transcription (Tye *et al.* 2019). Chaperones sequestered by the aggregated excess ribosomal subunit were believed to induce Hsf1p and consequent transcription of *BTN2*, *HSP42*, and *HSP82*, among other genes. While there is a slight increase of Hsp42 in our incurable mutants, there is no change of Hsp82 (Table 9) unlike the dramatic increase seen by Tye *et al.* (2019). Moreover, the effect observed by Tye *et al.* would be expected to facilitate [URE3] curing, rather than impair it. We also looked for other proteins involved in [URE3] propagation that showed substantial change in *rpl4aΔ* and *rpl21bΔ*, but not in *rps30aΔ*. The SILAC data does not show differential depression or elevation of any such proteins, including several that are Hsf1p-controlled (Table 9). However, it remains possible that Hsp42 capture by aggregates of ribosomal components plays a part in limiting the action of Btn2p or Cur1p.

A proteomic study of mutants deficient in a series of 60S or 40S subunit proteins found that no specific class of proteins was differentially affected in 40S subunit protein mutants, but that the ubiquitin/proteasome system (UPS) components were overproduced specifically in 60S subunit protein mutants (Cheng *et al.* 2019). Ubr2p is part of the cytoplasmic Mub1-Ubr2-Rad6 E3 ubiquitin ligase complex that targets Rpn4p, a transcription factor that promotes expression of genes for components of the UPS (Wang *et al.* 2004). Thus, *ubr2Δ* and *rplxΔ* strains should also have a hyperactive UPS system, and this effect should be blocked in *ubr2Δ rpn4Δ* and *rplxΔ rpn4Δ* strains, as we have observed. Hyperactivity of the UPS system could thus explain the effects of both *rplxΔ* and *ubr2Δ* mutants.

Btn2p and Cur1p are normally present at very low levels, but are elevated on inhibition of the UPS system (Malinovska *et al.* 2012; H. K. Edskes *et al.*, unpublished). Our western blots of Btn2p and Cur1p showed that overproduction of these proteins was diminished in *ubr2Δ* strains, with levels restored by an *rpn4Δ* mutation, indicating that effects on curing by Btn2p and Cur1p is mediated by the effects of *ubr2Δ* on the ubiquitin-proteasome system. The levels of overexpressed Btn2p and Cur1p were not significantly reduced in *rpl21bΔ* or *rpl4aΔ*. However, the effect of the *rplxΔ* mutations on curing was eliminated by *rpn4Δ*, again implicating the proteasome. It is possible that another factor involved in Btn2p and Cur1p overproduction curing is affected by proteasome activity. We also examined the levels of Btn3p, Sis1p, and Hsp42, all known to affect prion curing by Btn2p or Cur1p, but none were substantially altered in *rplxΔ* or *ubr2Δ* strains.

Our proteomic analysis of *rpl4aΔ* and *rpl21bΔ* strains by SILAC mass spectrometry revealed a specific dramatic elevation of Tma10p in these strains, but not in an *rps30bΔ* strain. However, co-overexpression of Tma10p from the very strong *GAL1* promoter did not impair curing of [URE3] by Btn2p. This implies that Tma10p elevation is not sufficient for the diminished curing, but

does not rule out some role for this protein. We also found that Tma10p is not necessary for [URE3] propagation. While several modestly over- or under-produced proteins were detected by the SILAC experiments (Supplementary Table S2), none had obvious connection to the curing process. The unamplified Btn2p levels in these strains were too low to be reliably detected by this method. It is possible that the protein(s) mediating the mutant effects on curing of [URE3] are not detected by the SILAC experiment.

Roles of Btn3 and Hsp42

Hsp42 overproduction can cure [URE3] and is necessary for overproduction curing by Btn2p or Cur1p (Wickner *et al.* 2014). Btn3p is an inhibitor of Btn2p for both its protein sorting and [URE3]-curing activities (Kanneganti *et al.* 2011). Btn3p directly binds to Btn2p and changes its localization, explaining the other effects (Kanneganti *et al.* 2011). We find that Btn3 is substantially elevated in *hsp42Δ*, a finding that could explain, in part, the lack of [URE3] curing by elevated Btn2p in *hsp42Δ* strains (Wickner *et al.* 2014). We also noted that Btn2-RFP is not co-localized with Ure2-GFP in [URE3-1] *hsp42Δ* strains, consistent with Btn3p blocking Btn2p action in this strain. Alternatively, Hsp42, which is known to bind to aggregated proteins (Specht *et al.* 2011), may be part of a Btn2-Hsp42-amyloid complex in which Btn2 [human HOOK1 homolog (Kama *et al.* 2007)] attaches to the cytoskeleton or microtubules to direct the complex to its special site.

Overall, our results suggest that large ribosomal protein gene mutations or *ubr2* mutation, through their known activation of proteasomes, increase the degradation of Btn2p or Cur1p or their cofactors, resulting in failure of curing of the [URE3] prion. Current work on a variety of human amyloidoses is revealing that they have many close similarities to the classical PrP-based prion diseases. *S. cerevisiae* has a wide array of anti-prion systems, blocking prion generation, curing most prion variants as they arise and reducing the toxicity of those that do arise, in each case without overexpression or deficiency of any components (reviewed in Wickner *et al.* 2018). These systems are analogous to the innate immunity systems of mammals, active against virus or bacterial infection. It is hoped that detailed understanding of these systems in yeast will facilitate the discovery and utilization of analogous or homologous systems in humans active against amyloid diseases. A region of Btn2p is homologous to human HOOK1 (Kama *et al.* 2007), a microtubule-binding protein involved in clathrin-independent endocytosis (Maldonado-Baez *et al.* 2013) and associated with tau aggregates in Alzheimer's disease (Herrmann *et al.* 2015). The association of the E3 ubiquitin ligases, *HECTD2* and *PARKIN* with susceptibility to PrP-based prion disease and Parkinson's disease (Shimura *et al.* 2000; Lloyd *et al.* 2009), respectively, is an interesting parallel with our findings.

Conflicts of interest

None declared.

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