Curing of the [URE3] prion by Btn2p, a Batten disease-related protein

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[URE3] is a prion (infectious protein), a self-propagating amyloid form of Ure2p, a regulator of yeast nitrogen catabolism. We find that overproduction of Btn2p, or its homologue Ypr158 (Cur1p), cures [URE3]. Btn2p is reported to be associated with late endosomes and to affect sorting of several proteins. We find that double deletion of *BTN2* and *CUR1* stabilizes [URE3] against curing by several agents, produces a remarkable increase in the proportion of strong [URE3] variants arising *de novo* and an increase in the number of [URE3] prion seeds. Thus, normal levels of Btn2p and Cur1p affect prion generation and propagation. Btn2p–green fluorescent protein (GFP) fusion proteins appear as a single dot located close to the nucleus and the vacuole. During the curing process, those cells having both Ure2p–GFP aggregates and Btn2p–RFP dots display striking colocalization. Btn2p curing requires cell division, and our results suggest that Btn2p is part of a system, reminiscent of the mammalian aggresome, that collects aggregates preventing their efficient distribution to progeny cells.

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Introduction

The infectious proteins (prions) of yeast, [URE3] and [PSI⁺], are self-propagating amyloid forms of Ure2p and Sup35p, respectively (Wickner, 1994; King and Diaz-Avalos, 2004; Tanaka *et al*, 2004; Brachmann *et al*, 2005). Both amyloids are parallel in-register β -sheet structures (Shewmaker *et al*, 2006; Baxa *et al*, 2007).

The generation and propagation of these amyloids are critically affected by many chaperones and their auxiliary factors. The disaggregating chaperone Hsp104 is necessary for both prions, and its overproduction leads to the loss of [PSI⁺] (Chernoff *et al*, 1995; Moriyama *et al*, 2000). The soluble cytoplasmic Hsp70s (Ssa proteins) affect the propaga-

tion of both prions (Jung *et al*, 2000; Roberts *et al*, 2004) and protect [PSI⁺] from curing by overproduction of Hsp104 (Newnam *et al*, 1999). Overexpression of Hsp40s destabilizes both [URE3] (Moriyama *et al*, 2000) and in some cases [PSI⁺] (Kushnirov *et al*, 2000). Hsp104, in cooperation with Hsp70s and Hsp40s disaggregate proteins (Glover and Lindquist, 1998), and these chaperones apparently function together to break long amyloid filaments into shorter ones, thereby creating new ‘seeds’ to propagate the prions (Kushnirov and Ter-Avanesyan, 1998). Among other chaperones affecting [PSI⁺] propagation are the ribosome-associated Hsp70s, called Ssb’s (Chernoff *et al*, 1999); several co-chaperones of Hsp70s, such as Sti1p and Cpr7p (Jones *et al*, 2004) and the nucleotide exchange factors for Hsp70s, Fes1p and Sse1p (Jones *et al*, 2004; Fan *et al*, 2007; Kryndushkin and Wickner, 2007). The effects of many of these factors on Hsp70s, and mutations of Hsp70s, indicate that the stabilization of the ATP-bound form of Ssa1p promotes [PSI⁺] propagation, whereas the ADP-bound form destabilizes [PSI⁺] (Jones *et al*, 2004). For [URE3], the alteration of Ssa1 activity in either direction can damage prion propagation (Kryndushkin and Wickner, 2007), suggesting that the normal level of Hsp70 activity is required for [URE3].

Other factors affecting prion propagation in yeast include cytoskeletal proteins (Ganusova *et al*, 2006) and elements of the ubiquitin system (Allen *et al*, 2007). In addition, [PSI⁺], and to a lesser extent [URE3], prion generation is promoted by another prion called [PIN⁺] (for *P*si *i*nducibility), which is an amyloid of the Rnq1 protein (Derkatch *et al*, 2001; Bradley *et al*, 2002).

Here, we identify Btn2p and its homologue, Ypr158p (Cur1p) as proteins able to destabilize [URE3]. We find that Btn2p curing of [URE3] involves colocalization of Ure2p prion aggregates and the Btn2 protein. Moreover, normal levels of Btn2p and Cur1p lower [URE3] prion seeds and affect the spectrum of prion variants arising.

Results

Identification of Btn2p and Ypr158p (Cur1p) as factors that can destabilize [URE3]

We recently described a genomic screen for prion-eliminating factors (Kryndushkin and Wickner, 2007). Strain BY241, bearing *ADE2* under control of the *DAL5* promoter (Brachmann *et al*, 2005), was used to monitor the activity of Ure2p. Prion inactivation of Ure2p allows *ADE2* transcription, resulting in pink or white colonies according to the level of transcription, whereas active Ure2p makes such a strain Ade[–] and red on adenine-limiting medium. Utilizing this system, we showed that overproducing the chaperones Ydj1p and Sse1p could cure [URE3] (Kryndushkin and Wickner, 2007). Here, we show that overproduced Btn2p and Ypr158p (which we name Cur1p for ‘Curing of [URE3]’) are also prion-curing factors. Overexpression of either *BTN2* or *CUR1* in the strain BY241 [URE3] v1 (a mitotically stable [URE3] prion

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Figure 1 Overexpression of *CUR1* and *BTN2* cures [URE3]. (A) Transformants of BY241 [URE3] v1 or [ure-o] (lacking [URE3]) with multicopy plasmids carrying the indicated genes. Controls (C): pRS425 vector; +: centromeric plasmid; +++: high-copy plasmid and native promoters; ++++: high-copy plasmid and *TEF2* promoter. (B) Expression levels of Hsp70s, Sse1p and Ydj1p are not altered by overproduction of Btn2p or Cur1p. Lysates of BY241 [ure-o] wild-type, *btn2 Δ cur1 Δ* and overproduced Btn2p, Ydj1p, Sse1p, Cur1p and Ssa1p cells were analysed by immunoblotting with antibodies against Sse1p, Hsp70s or Ydj1p. Act1p detection was used as a loading control.

Table I Relative prion loss after overproduction of 'anti-prion' factors

Overproduced proteins	None	Btn2p	Cur1p	Ssa1p	Ydj1p
Relative BY241 [URE3] v1, loss (%)	1	65	60	20	50
Relative BY251 [URE3] loss (%)	1	55	60	15	50

Ssa1p and Ydj1p are known to cure [URE3] (included for comparison). Data shown are the average of four experiments, with a variation of about 10%.

variant) results in approximately 70% prion loss among transformants grown for 3 days (Figure 1A; Table I; see Materials and methods). It is noted that the percentage is increased after additional growth of transformants. Similar curing was obtained with two other prion variants in the same host (100 and 70%) and with the same variant with two other hosts (55 and 80%). These proteins are about 45% similar (28% identical) with no detectable homology to known chaperones.

As chaperones were the only known cellular factors interfering with yeast prions after overproduction, our finding may correspond to a new mechanism of prion elimination.

Despite the lack of homology to chaperones, Btn2p and Cur1p may be involved in the cellular stress response, because each is induced by various types of stress conditions including heat shock, starvation, stationary phase and others (Supplementary Table I). Indeed, the promoters of *BTN2* and *CUR1* share HSEs (heat shock elements) with chaperone promoters. However, Btn2p or Cur1p overproduction does not induce significant changes in the expression levels of Ure2p (data not shown) or of chaperones, the overproduction of which is known to cure [URE3] (Ssa1p, Ydj1p and Sse1p) (Figure 1B). Therefore, it seems unlikely that the curing effect of Btn2p and Cur1p is due to upregulation of chaperones.

The efficiency of 'anti-prion' Btn2p action is strongly dosage dependent: although one additional copy of *BTN2* on a centromeric plasmid results only in a few small red sectors in a number of transformants, vigorous overexpression of *BTN2* under the *TEF2* promoter on a high-copy plasmid leads to almost complete curing (Figure 1A).

Btn2p has no homologues in the yeast proteome except Cur1p, but in mammals Hook proteins are homologous to Btn2p (Kama *et al*, 2007, see also Discussion). We expressed human Hook1 and Hook2 proteins in BY241 [URE3] v1 under the control of either *TEF2* or *GAL1* promoters and tested whether they can cure [URE3] v1. No detectable curing was found by either protein (data not shown).

Deletions of both *BTN2* and *CUR1* lead to prion stabilization and increased induction rates for strong variants of [URE3]

Deleting either *BTN2* or *CUR1* or both genes in strain BY241 [URE3] v1 did not destabilize [URE3] (Figure 2A); in contrast, we observed a stabilizing effect of the deletions in many conditions that resulted in [URE3] loss such as 10% DMSO treatment, acid shock (0.035% HCl), 0.2 mM latrunculin A treatment (Bailleul-Winslett *et al*, 2000), as well as overexpression of prion-curing chaperones Ssa1p and Hsp104p (summarized in Table II). The most substantial effect was found with the double deletion strain, in agreement with the fact that Btn2p and Cur1p are homologous.

We also found that deletion of either *BTN2* or *CUR1* or both produced a mild increase in the frequency of *de novo* [URE3] appearance (Table III). Remarkably, although most newly induced [URE3] variants were weak and unstable in the wild-type strain, we found a substantial relative increase in the proportion of stable strong [URE3] variants (large white colonies) in the *btn2 Δ cur1 Δ* double deletion strain (Figure 2B; Table III). The strong [URE3] phenotype remained after crossing of large white [URE3] clones with wild-type [ure-o] cells indicating that the [URE3] variant is strong, independent of the *btn2 Δ cur1 Δ* background. The above data suggest that normal levels of Btn2p and Cur1p affect both prion generation and propagation.

Cellular localization of Btn2p and Cur1p

Cellular localization of Btn2p remains controversial. Although one group showed cytoplasmic localization for this protein (Chattopadhyay *et al*, 2000), a recent paper from Kama *et al* (2007) claimed colocalization of Btn2p with late endosomal markers. We reproduced the results of the latter report finding punctate localization of Btn2p-green fluorescent protein (GFP) (using the plasmids from Kama *et al*), as well as GFP-Btn2p (using genomic replacement of

Figure 2 Deletions of *BTN2* or *CUR1* do not destabilize [URE3] and lead to increased induction rates for strong variants of [URE3]. (A) BY241 [ure-o] and [URE3] wild-type strains and with the indicated deletions were streaked on $\frac{1}{2}$ YPD. (B) BY241 [ure-o] wild-type and *btn2Δcur1Δ* cells were transformed with pH376-Ure2. Following the expression of Ure2p, about 10^6 cells were spread on plates lacking adenine to select [URE3]. Photographs were taken after 8 days of growth.

Table II Stability of BY241 [URE3] v1 after growth in different conditions

	WT	<i>btn2Δ</i>	<i>cur1Δ</i>	<i>btn2Δcur1Δ</i>
No treatment	0.1	0.1	0.1	<0.1
DMSO treatment	20	14	8	1
Acidic shock (0.035% HCl)	35	25	5	3
Overexpression of <i>SSA1</i>	15	16	12	4
Overexpression of <i>HSP104</i>	8	ND	ND	0.5
Latrunculin A treatment	15	ND	ND	0

Data shown are the percentage of cells that have lost the prion and represent the average of three independent experiments with standard variation being about 10% (see Materials and methods for details).

the wild-type gene in strain BY241 [URE3] with *GAL1* promoter–GFP–*BTN2*). Both GFP–Btn2p and Btn2p–GFP were functional, as they were able to cure [URE3] with similar efficiency as Btn2p itself (data not shown). Both hybrids behaved similarly and could be detected usually as one or two dots per cell close to the nucleus (Figure 3A and B). However, under strong overexpression under the *TEF2* promoter on a high-copy plasmid, Btn2p–GFP is easily detected in the cytoplasm as well (Figure 3A).

To determine the domain(s) of Btn2p responsible for the prion curing and localization, we made two N-terminal deletions in Btn2p–GFP (resulting in amino acids 54–410 or 100–410 of Btn2p fused to GFP) and two C-terminal ones in GFP–Btn2p (GFP fused to either amino acids 1–233 or 1–277

Table III Generation of strong [URE3] variants is enhanced in *btn2Δcur1Δ* cells

Host	Total ADE+ colonies per 10^6 cells	Big white [URE3] colonies per 10^6 cells ^a
Wild type	140	1
<i>btn2Δ</i>	195	5
<i>cur1Δ</i>	285	12
<i>btn2Δcur1Δ</i>	210	20

^aNumbers represent ADE+ colonies with [URE3] that were confirmed by GuHCl curability (see Materials and methods). As most small ADE+ clones were unstable, only big clones were tested and counted.

The wild-type (BY241 [ure-o]), BY241 *btn2Δ::TRP1*, BY241 *cur1Δ::KanMX4* and BY241 *btn2Δ::TRP1 cur1Δ::KanMX4* strains containing *URE2* driven by galactose-inducible promoter on episomal plasmid were tested. After 2 days of induction on galactose in liquid culture, 10^6 yeast cells were spread on standard SC plates without adenine. The average number of colonies formed after 5 days of incubation at 30°C is shown (the data from three independent experiments were combined; standard variation was about 20%).

Figure 3 Cellular localizations of Btn2p and Cur1p. (A) Expression of Btn2-GFP from a single-copy plasmid (pRS316-Btn2-GFP) and from a high-copy plasmid (pH400tef2-Btn2-GFP). (B) Perinuclear localization of Btn2-RFP (expressed from pRS316-Btn2-RFP) is shown by comparison with nucleoplasmic protein GFP-Pus1. (C) The colocalization of GFP-Cur1 and RFP-Pus1 indicates the nuclear localization for Cur1p.

of Btn2p). On overexpression, each of these mutants could cure BY241 [URE3] v1 with varying efficiency, but residues 100–233 could not (Table IV). Interestingly, some mutants showed multiple dots per cell instead of the one or two seen with the full-length protein (Supplementary Figure 1).

Unlike Btn2p, Cur1p shows nuclear localization (Figure 3C) as determined in strain BY241 [URE3] *GAL1* promoter–GFP–*CUR1*. This unexpected finding indicates that despite the homology between these proteins, the actual mechanisms underlying the curing effect may be different. Alternatively, they both may partially escape their normal localization on overproduction and affect [URE3] propagation in the cytosol.

Table IV Curing (%) of BY241 [URE3] v1 by deletion derivatives of Btn2p-GFP

Time (h)	54–410 aa	100–410 aa	1–233 aa	1–277 aa	100–233 aa	1–410 aa (wild type)
0	1	0.5	0.5	0.5	0.5	1
13	27	10	20	4	0.5	30
30	52	25	50	8	0.5	60

Either wild-type Btn2p or Btn2p deletion mutants fused with GFP were overproduced under a GAL1 promoter in BY241 [URE3] v1. Cells were spread on $\frac{1}{2}$ YPD plates and prion loss was counted. The first row represents the coded amino acids for each Btn2p mutant; the first column shows the time of induction on galactose and the table contains the percentage of cured cells for each combination. The data from two independent experiments were combined; standard variation was about 15%.

Btn2p is present in high molecular weight complexes and does not associate with Ure2p monomers

We assume that Btn2p is a cytosolic endosome-associated protein that interacts with the endocytic SNARE complex and other proteins. Indeed, we were unable to find any signal peptide sequence within Btn2p (or Cur1p), and Kama *et al* (2007) found no change in Btn2p localization in strains defective in intracellular protein sorting, such as vps23Δ, vps27Δ, sec21-2, rcy1Δ and many others. Finally, we did see clear cytoplasmic staining after strong overexpression of Btn2p-GFP (Figure 3A).

To test whether Btn2p interacts directly with [URE3] cytoplasmic aggregates, we performed immunoprecipitation experiments with lysates of BY241 [URE3] and [ure-o] (the absence of [URE3]) cells expressing either Btn2p-GFP or GFP-Btn2p. After precipitation of Ure2p using Ure2p C-terminal antibodies, we did not detect Btn2p-GFP fusions with antibodies against GFP (data not shown). This may be explained as the lack of interaction between Btn2p and Ure2p or that technical problems interfered with the detection. First, we found Btn2p in a high molecular weight complex, which can be sedimented efficiently at 16 000 g (Figure 4A–C), was resistant to NP-40 treatment (data not shown) and may represent the published association of Btn2p with endocytic SNAREs and other proteins (Kama *et al*, 2007). Second, [URE3] prion aggregates are themselves >3 Mdal (Kryndushkin and Wickner, 2007), so the detection of potential Btn2p–Ure2p interaction by immunoprecipitation may be challenging.

We also tested the effects of Btn2p on the assembly of Ure2p into amyloid fibres *in vitro*. For this purpose, we expressed recombinant Btn2p under native conditions as a fusion with glutathione S-transferase (GST). As controls, we used recombinant GST and Ydj1p; the latter modestly inhibits the Ure2p polymerization rate *in vitro* (Lian *et al*, 2007). Full-length recombinant Ure2p, purified under native conditions, was mixed with two-fold excess of GST, GST-Btn2p or Ydj1p. Amyloid assembly of Ure2p was monitored using thioflavin-T binding (Figure 4D). As expected, Ydj1p had an inhibitory effect on the assembly reaction. However, we did not detect a substantial difference between GST and GST-Btn2p (Figure 4D). Together with our immunoprecipitation data, it indicates that *in vivo* Btn2p does not bind Ure2p monomers when promoting [URE3] loss, but rather must function on the level of prion fibres.

Colocalization of Btn2p with [URE3] prion aggregates during the curing process

To understand the mechanism of [URE3] curing by overproduction of Btn2p, we used confocal microscopy to trace the prion aggregates. Strain BY241 [URE3] v1 was trans-

Figure 4 Btn2p is present in high molecular weight complexes and has little effect on the assembly of Ure2p into amyloid fibres. (A) Centrifugation analysis of BY241 [ure-o] carrying centromeric (cen) or high-copy (h.c.) plasmids expressing Btn2-GFP. T, total lysate; S, supernatant fraction; P, pellet fraction; $k = 1000$ g. Fractions were analysed by western blot with anti-GFP antibodies. The distribution of cytosolic proteins, Hsp70s (B) and Act1p (C), was also detected in the same fractions with corresponding antibodies. (D) Kinetics of Ure2p amyloid formation. Thioflavin-T was used as a reporter of Ure2p amyloid formation. Ure2p (10 μ M) was incubated with 20 μ M GST, GST-Btn2p or Ydj1p, with constant stirring at room temperature. The normalized fluorescence change is presented ($\lambda_{\text{ex}} = 420$ nm; $\lambda_{\text{det}} = 480$ nm).

formed with two plasmids: pVTG12, a centromeric plasmid with URE2^{1–89}-GFP to decorate [URE3] aggregates (Edskes *et al*, 1999) and pYES52-Btn2-RFP, a high-copy plasmid bearing BTN2-red fluorescent protein (RFP) under the control of the inducible GAL1 promoter. The expression of pVTG12 itself did not result in [URE3] curing (Edskes *et al*, 1999). After overnight growth on raffinose-containing medium, cells were transferred to a medium with 2% galactose and 1% raffinose for the induction of Btn2p-RFP expression. During the induction, we followed the behaviour of [URE3]

aggregates as well as Btn2p–RFP dots by confocal microscopy and at the same time checked the extent of [URE3] curing by spreading cells on glucose-containing media and counting the percentage of red colonies. After 24 h of induction, about half of the cells still contained detectable [URE3] aggregates, whereas the other half had diffuse fluorescence indicative of prion loss. Interestingly, in most cases cells contained either Ure2N–GFP prion aggregates or Btn2p–RFP dots but not both (Supplementary Figure 2). This probably reflects the fact that cells with elevated Btn2p–RFP amount lost the prion faster. However, some cells (several percentage of total population) still contained both aggregates and dots. In such cells, we found colocalization of Btn2p–RFP with prion aggregates (Figure 5A; Supplementary Figure 3). Importantly, colocalization could be observed only when prion curing became efficient. [URE3] curing reflects the accumulation of Btn2p–RFP (Figure 5B), reaching about 50% only after 24 h of induction. Consistent with curing rates, colocalization was occasional after 7 h of induction (10–15% of curing) and became frequent after 13–15 h of induction (about 30% of curing).

The same colocalization experiment was performed with GFP–Cur1p, but unlike Btn2p, Cur1p was visible only in nuclei of yeast cells (Figure 3C), so no colocalization between GFP–Cur1p and [URE3] prion aggregates was detected (data not shown). Sequence homology between Btn2p and Cur1p suggests a similar mechanism for prion curing. It is still possible that curing of [URE3] by Cur1p occurs with a minor fraction of Cur1p that escapes the nucleus on overproduction but is not detectable by fluorescent microscopy. Nuclear localization of this protein may suggest a role of Cur1p in transcription regulation, so it might exert an effect on [URE3] through known prion-curing chaperones or Btn2p. However, a *btn2Δcur1Δ* strain can be cured by the overexpression of either *BTN2* or *CUR1* with similar efficiency as a wild-type strain (data not shown). Also, we did not find alterations in chaperone levels after Cur1p overproduction (Figure 1B). Overall, the mechanism underlying Cur1p action on [URE3] remains to be clarified.

Cell division is necessary for curing of [URE3] by Btn2p

Prion loss may occur through active degradation of prion particles or by retention of prion seeds during cell divisions. In the latter case, the prion will remain in only one of the two progeny cells but absent in the other and will soon be eliminated from the population. To find out how Btn2p exerts an effect on [URE3], we followed the kinetics of [URE3] curing in the presence of alpha factor that stops cell division. The addition of 50 μ M alpha factor to the growth media during the induction drastically reduced the efficiency of [URE3] curing close to the control level, but still preserved the colocalization (Figure 5B and C). This situation is also favourable for detecting possible interaction between [URE3] aggregates and Btn2p because the proportion of cells containing both the excess of Btn2p and [URE3] is elevated. Indeed, in the presence of alpha factor, we were able to detect a weak interaction between Ure2p and GST–Btn2p (Supplementary Figure 5).

Taken together, these findings strongly indicate that cellular division is essential for elimination of [URE3] by Btn2p overproduction. Btn2p may facilitate the aggregation of prion

Figure 5 Colocalization of Btn2p with [URE3] prion aggregates during the curing process. (A) BY241 [URE3] v1 was transformed with pVTG12 to decorate prion aggregates and pYES52–Btn2–RFP for Btn2p–RFP induction. Efficient colocalization was observed during 15–24 h of induction and visualized by fluorescence confocal microscopy. (B) Time course of [URE3] curing for the strain from (A) grown in the presence of raffinose, galactose or galactose and alpha factor. (Inset) The expression level of Btn2p–RFP during induction; cen: Btn2p–RFP expressed from pRS316–Btn2–RFP. (C) The same strain was grown in the presence of galactose and alpha factor and subjected to confocal microscopy. ‘Schmoo’ morphology was observed after the addition of alpha factor.

seeds at one site preventing their efficient distribution and so causing prion loss in the cell population.

Effects of Btn2p and Cur1p overproduction on other yeast amyloids

To further characterize the curing effect of Btn2p/Cur1p proteins, we examined whether Btn2p or Cur1p overproduction can destabilize other known yeast prions: [PSI⁺] and [PIN⁺]. We also tested [PSI_{PS}⁺], a prion based on a *Pichia–Saccharomyces* (Sup35–PS) hybrid. [PSI_{PS}⁺] variant 1, in contrast to [PSI⁺], can be cured by either Ydj1p or Sse1p

overexpression (Kryndushkin *et al*, 2002; Kryndushkin and Wickner, 2007), resembling [URE3] rather than [*PSI*⁺]. We found that the action of Btn2p was [URE3] specific; none of the other prions was destabilized by Btn2p overproduction (data not shown). However, overexpression of Cur1p had a modest destabilizing effect on the propagation of [*PSI*_{PS}⁺] no. 1 (about 15% curing when compared with 1% for the control plasmid), but not [*PSI*⁺] or [*PIN*⁺].

We examined the cellular distribution of Sup35 prion aggregates and Btn2p dots by performing similar colocalization experiments as were described above. [*PSI*⁺] aggregates were visualized with a Sup35NM-GFP plasmid and the expression of Btn2p-RFP was induced with a *GAL1* promoter. Surprisingly, despite the absence of [*PSI*⁺] curing, we observed a high level of colocalization between Btn2p-RFP and [*PSI*⁺] aggregates (Figure 6A). To find more general effects, we expressed in yeast cells the GFP-tagged huntingtin exon 1 fragment encoding the long polyglutamine stretch (103 Q) under a *GAL1* promoter. This protein is widely used to model intracellular polyglutamine aggregation and toxicity (Meriin *et al*, 2002, 2007). Following overproduction, it formed several foci, observed by fluorescent microscopy (Figure 6B). In this case, we found only partial colocalization of Btn2p-RFP and Q103-GFP, perhaps due to the vigorous aggregation Q103-GFP and partial distribution of Q103-GFP to the nucleus (Sokolov *et al*, 2006).

Figure 6 Colocalization of Btn2p with [*PSI*⁺] prion aggregates and polyglutamine aggregates. (A) 5V-H19 [*PSI*⁺] was transformed with 181-Sup35NM-GFP to decorate prion aggregates and pYES52-Btn2-RFP for Btn2-RFP induction. Efficient colocalization was observed during 15–24 h of induction and visualized by fluorescence confocal microscopy. (B) BY241 [ure-o] cells carrying pRS425-Btn2-RFP and pQ103-GFP were grown in the presence of galactose for 4–6 h to induce Q103-GFP expression. After that, cells were visualized by fluorescence confocal microscopy.

These data suggest a common place for amyloid aggregates inside yeast cells, marked by Btn2p localization. We propose that this compartment collects aggregates and limits their distribution to progeny cells on cell division as we see colocalization of Btn2p with [URE3] aggregates and the curing process requires cell division.

Fewer propagons in [URE3] may explain the specificity of Btn2p action

We estimated [URE3] prion seeds by the method developed for [*PSI*⁺] (Eaglestone *et al*, 2000), blocking seed/propagon formation with the Hsp104 inhibitor, guanidine HCl, and following dilution of seeds as cells divide by the appearance of seedless (prion-less) progeny. We assume that the seed number is halved at each generation after guanidine addition. Strain BY241 [URE3] v1 was transferred during log phase into YPD medium supplemented with 3 mM GuHCl; samples were taken at various intervals and were spread on plates to count the percentage of [URE3] loss. In parallel, BY241 [URE3] *btn2Δcur1Δ* and two independent [*PSI*⁺] strains were used for comparison. The curing profile for [URE3] was much faster when compared with the two [*PSI*⁺] strains (Figure 7). The double deletion strain showed a slower curing profile, which is consistent with observed [URE3] stabilization (see above). If Btn2p participates in the retention of prion seeds at some site, preventing the efficient distribution of seeds during cellular division, then the efficiency of Btn2p action will be greatly dependent on the number of propagons. On the basis of curing profiles, we estimate the number of propagons at 34 for [URE3], 97 for [URE3] *btn2Δcur1Δ*, 1180 for 5V-H19 [*PSI*⁺] and 1550 for strain 74-D694. The smaller propagon number for [URE3] may explain the specificity in Btn2p action and may arise from the lower expression level of Ure2p when compared with Sup35p (estimated as 7060 versus 78 900 molecules per cell, respectively (Ghaemmaghani *et al*, 2003). The propagon numbers for [*PSI*⁺] strains were not changed significantly after overpro-

Figure 7 [URE3] cells contain less propagons than [*PSI*⁺] cells. BY241 [URE3] wild-type or *btn2Δcur1Δ* as well as 5V-H19 and 74-D694 [*PSI*⁺] cultures were grown logarithmically in YPD with 3 mM GuHCl. The percentages of prion-positive colonies recovered following plating on $\frac{1}{5}$ YPD have been plotted as a function of generations, which have been calculated from OD₆₀₀ and colony counts. The 63.2% line was used to estimate propagon numbers.

duction of Btn2p (data not shown), consistent with the absence of [PSI⁺] curing.

Autophagy may be involved in managing of [URE3] prion aggregates but is not required for [URE3] curing by *BTN2* overexpression

As Btn2p may participate in vacuolar trafficking (Kama *et al*, 2007), we tested whether it may be involved in autophagy, a non-selective process by which cytoplasmic constituents are turned over (Yorimitsu and Klionsky, 2005). Autophagy is the main route for degradation of large misfolded protein aggregates inside the cell (reviewed in Rubinsztein, 2006) and it seems reasonable to test whether Btn2p mediates its effect through the degradation of prion aggregates in the vacuole (the yeast lysosome). In addition, autophagy is disrupted in mouse models of Batten disease (Cao *et al*, 2006) and overexpression of Btn2p in yeast may represent a compensatory mechanism for this defect.

First, we tested whether the induction of autophagy can cure [URE3]. After growth in normal SC medium, BY241 [URE3] v1 cells were washed and transferred to a nitrogen-poor SD(−N) medium that is widely used for autophagy induction (Shintani and Klionsky, 2004). After several days of starvation, cells were spread on $\frac{1}{2}$ YPD and observed by colour. No destabilization of the prion was detected (data not shown). This strongly argues that autophagy does not interfere with [URE3] prion propagation, although it did help mammalian cells to degrade aggregated huntingtin and ataxin-1 proteins (Iwata *et al*, 2005). In these experiments, BY241 *btn2Δcur1Δ* [URE3] cells had reduced survival under starvation conditions when compared with wild-type BY241 [URE3] or individual deletions (Supplementary Figure 4), suggesting that Btn2p or Cur1p is required for normal survival under starvation conditions. To monitor the induction of autophagy, we analysed the processing of a GFP–Atg8 hybrid protein. GFP–Atg8 is transported into the vacuole inside autophagosomes and degraded; however, the GFP itself is relatively resistant to proteolysis (Shintani and Klionsky, 2004). *ATG1* encodes a protein kinase required for autophagy (Shintani and Klionsky, 2004). We were able to detect free GFP protein in wild-type and *btn2Δcur1Δ* lysates, but not in the *atg1Δ* lysate of cells, expressing GFP–ATG8, indicating that Btn2p and Cur1p are not required for autophagy (Supplementary Figure 5).

Further, we asked whether the curing effect of Btn2p was autophagy dependent. Btn2p–GFP was expressed under the control of *GAL1* promoter in wild-type and autophagy-deficient (*atg1Δ*) [URE3] cells and curing rates were compared (Supplementary Figure 6). We observed only a slight reduction in the curing efficiency for [URE3] *atg1Δ*. Thus, autophagy is not required for [URE3] curing by *BTN2* overexpression.

Finally, we tested whether autophagy can affect the induction of [URE3]. We compared [URE3] induction rates of a wild-type strain with autophagy-deficient *atg1Δ* and *atg8Δ* strains and found a mild (about two-fold) increase in *de novo* [URE3] appearance in strains with *atg* deletions (data not shown). This indicates that autophagic proteins may be involved in managing of prion aggregates; however, additional studies are required to address this question more thoroughly.

Discussion

New function of Btn2p

Previously, only overproduced chaperones had been found to interfere with the propagation of [URE3] or [PSI⁺], so Btn2p and Cur1p constitute a new class of prion-curing factors in yeast. Both proteins are predicted to have a coiled-coil structure and contain a high proportion of charged residues. They are homologous to each other and to Hook proteins of higher eukaryotic organisms (Kama *et al*, 2007).

Juvenile neuronal ceroid lipofuscinosis (Batten disease) is a recessive inherited neurodegenerative disorder characterized by the accumulation of autofluorescent material in the cell lysosomes of afflicted individuals. The disease is caused by mutations in the *CLN3* gene, encoding a lysosomal transmembrane protein that is conserved from yeast to humans. Because yeast *BTN1* is a *CLN3* orthologue, *btn1Δ* mutants model Batten disease (Pearce *et al*, 1999). Btn1p regulates vacuolar pH and the elevation of Btn2p in response to *BTN1* deletion may represent a compensatory mechanism that returns the pH to normal. Btn2p is also necessary for proper localization of Rsg1p, a negative regulator of Can1p Arg-Lys permease; overexpression of Btn2p leads to decreased arginine uptake (Chattopadhyay and Pearce, 2002). That *btn2* mutants also mislocalize two other proteins indicates a role in cellular trafficking (Kim *et al*, 2005 and references therein). A recent report indicates that Btn2p functions in endosome–Golgi retrieval and demonstrates Btn2p binding to the endocytic SNARE complex (Kama *et al*, 2007). Our findings broaden the possible cellular roles of Btn2p and suggest a functional interaction between Btn2p and [URE3] prion aggregates.

Functional similarities between Btn2p and Hook proteins

The cytoplasmic Hook proteins bind to microtubules and to organelles, transporting them along the microtubules (Walenta *et al*, 2001). Hook1 in *Drosophila* is involved in the endocytosis of membrane receptors and their delivery to multivesicular bodies (Sunio *et al*, 1999). All three human Hook proteins share a conserved N-terminal microtubule-binding domain and an extended central coiled-coil motif, the region homologous to Btn2p. Further, Hook1 is elevated almost three-fold in tissue derived from a *CLN3*-knockout mouse (Weimer *et al*, 2005), just as Btn2p is elevated in *btn1Δ* yeast. In addition, a weak interaction between Hook1 and *CLN3* was also detected (Luiro *et al*, 2004).

Strikingly, overexpression of Hook2 promotes the formation of an aggresome from a model substrate, cystic fibrosis transmembrane regulator (Szebenyi *et al*, 2007). Hook2 localizes to the centrosome and may contribute to the positioning or formation of aggresomes that are pericentriolar accumulations of misfolded proteins. Hook2 overexpression enhanced the recruitment of aggresome components to the centrosome, suggesting that Hook2 may exert an effect on the dynein-mediated retrograde transport of misfolded substrates to the aggresome (Szebenyi *et al*, 2007).

Similarly, we show that overproduction of the Hook orthologue Btn2p also gathers misfolded substrates (prion aggregates), resulting in the loss of the prion. However, direct comparison should be taken with caution as human Hook

proteins did not directly substitute for Btn2p in our curing assays (see Results).

A model for Btn2 action

Our data suggest that Btn2p can interact with prion aggregates, preventing prion seeds from being transmitted to progeny cells. Prion loss in a yeast population, following the overexpression of a protein, could occur in several different ways. Such protein may interact and stabilize the normal conformation of the prion protein, thus destabilizing the prion fibres. Alternatively, it may function on the level of prion aggregates (prion seeds), sequestering them and preventing effective prion transmission during cellular division or helping to degrade them. The first mechanism looks unlikely for Btn2p, as we did not find an interaction between Btn2p and monomeric Ure2p in lysates and also Btn2p had little effect on Ure2p amyloid formation *in vitro*. In addition, Btn2p and Cur1p overproduction changed neither the level of Ure2p itself nor the level of chaperones that are known to interfere with [URE3] propagation. In addition, the curing rate was greatly reduced when cell division was stopped by alpha factor, indicating that prion aggregates were not efficiently degraded inside the cell. Also, we did not detect prion curing after autophagy activation, a mechanism by which mammalian cells get rid of intracellular protein aggregates in several cellular models of amyloid disorders (Iwata *et al*, 2005; Rubinsztein, 2006). Most likely, Btn2p sequesters [URE3] prion aggregates in a particular place and/or targets them to the vacuole for degradation (Figure 8). This model is supported by colocalization of Btn2p with Ure2p prion foci during prion loss as well as by possible interaction between Ure2p and Btn2p. Interestingly, we also found colocalization of Btn2p with Sup35p prion foci as well as with disease-associated Huntingtin aggregates, although we did not detect curing of [PSI⁺]. Yeast cells apparently have a certain place (the perivacuolar compartment marked by Btn2p), reminiscent of the mammalian aggresome, where intracellular amyloid aggregates may be sequestered. [PSI⁺] cells contain more prion seeds ('propagons') than [URE3] cells, and [URE3] SDS-resistant polymers from the strain used in this study are larger than [PSI⁺] polymers (Kryndushkin and Wickner, 2007). We speculate that the larger number of smaller Sup35p prion aggregates (seeds) exceeds the ability of even overproduced Btn2p to sequester them and accounts for the absence of [PSI⁺] curing.

Implications for Batten disease pathology

In mouse models of Batten disease, the accumulation of undegraded substrates in lysosomes disrupts autophagy probably through impairment of the fusion between autophagosomes and lysosomes (Cao *et al*, 2006). In tissue derived from these CLN3-knockout mice, Hook1, a homologue of Btn2p, is elevated (Weimer *et al*, 2005), just as Btn2 is elevated in *btn1Δ* yeast. Hook1 may be elevated because of the need to collect the debris from unsuccessful autophagy. We find that elevation of Btn2p leads to increased collection of prion aggregates in an aggresome-like site, and that Btn2p and Cur1p have effects at normal levels in the same direction as *btn2Δcur1Δ* mutants have increased seed number. Alternatively, pathologically elevated Hook1 may excessively collect materials that are directed to the lysosome, thus

Figure 8 A model for Btn2 function in managing of prion aggregates. (A) Wild-type cells: prion aggregates are normally distributed to progeny cells during cytokinesis. Btn2p interacts with some prion seeds, reducing their quantity. (B) Cells with overproduced Btn2p: Btn2p sequesters prion aggregates and prevents their efficient distribution to progeny cells. In addition, it may target prion aggregates to vacuolar degradation.

contributing to the accumulation of the lipofuscin of Batten disease.

Materials and methods

Yeast strains and media

Strains BY241 (Mat a, *ura3*, *leu2*, *trp1*, *P_{dal5}-ADE2*, *kar1*, [URE3] or [ure-o]) and BY251 (Mat α, *his3*, *leu2*, *trp1*, *P_{dal5}-ADE2*, *kar1*, [URE3] or [ure-o]) contain the *ADE2* gene under control of the *DAL5* promoter (Brachmann *et al*, 2005), allowing detection of the prion state of Ure2p and even different [URE3] prion variants by colony colour. For [PSI⁺] experiments, strains 74-D694 (Mat a, *ade1-14*, *ura3*, *leu2*, *trp1*, *his3*, [PSI⁺]) (Chernoff *et al*, 1995) or 5V-H19 (Mat a, *ura3*, *leu2*, *ade2-1*, SUQ5) with strong or weak prion variants (Kushnirov *et al*, 2000) were used. PS-5V-H19 [PSI_{PS}⁺], which expresses hybrid Sup35 with a prion domain from *Pichia methanolica* as a result of an altered chromosomal *SUP35* locus (Kushnirov *et al*, 2000), was checked for additional effects of Btn2p and Cur1p overproduction. *BTN2*, *CUR1*, *ATG1* and *ATG8* disruption cassettes were obtained by amplifying yeast genomic DNA of corresponding strains from the *Saccharomyces cerevisiae* knockout collection (Winzler *et al*, 1999) using primers: 5'-tggaagatctattg cattac-3' and 5'-tagcataaatgttaacatgg-3' for *BTN2*; 5'-gcaattgatagcgc cacac-3' and 5'-aacgcgctattagcgatgc-3' for *CUR1*; 5'-ttaatatcttct gatcgatc-3' and 5'-aaggatgatgatgccaagg-3' for *ATG1*; 5'-gttcaaattat gaaacaactc-3' and 5'-tactgtatcatctatttgc-3' for *ATG8*. Disrupted mutants were then obtained by transforming the resulting PCR fragment into yeast and selecting for G418-resistant colonies at a final concentration of 0.5 g/l. To make BY241 *btn2Δcur1Δ* strain, *TRP1* gene was amplified with primers that contained about 70 bp of *BTN2* promoter and terminator sequences. BY241 *cur1Δ* strain was transformed with such fragment and TRP⁺ clones were selected; most of them obtained *btn2Δ::TRP1*. In each case, disruption was confirmed by PCR. BY241 [URE3] with integrated *GAL1* promoter-GFP-*BTN2* (or *CUR1*) was made by homologous recombination as described by Longtine *et al* (1998) (wild-type genes were substituted with GFP fusions).

Table V Plasmids used in this study

Plasmid	Promoter	Marker	Copy number	Source
pRS425-Btn2	<i>BTN2</i>	Leu2	High copy	This study
pRS425-Cur1	<i>CUR1</i>	Leu2	High copy	This study
pRS425-Ssa1	<i>ADH1</i>	Leu2	High copy	Kryndushkin and Wickner (2007)
pFL44-Hsp104	<i>HSP104</i>	Ura3	High copy	Kushnirov <i>et al</i> (2000)
pRS316-Btn2-GFP	<i>ADH1</i>	Ura3	Centromeric	Kama <i>et al</i> (2007)
pRS316-Btn2-RFP	<i>ADH1</i>	Ura3	Centromeric	Kama <i>et al</i> (2007)
pRS425-Btn2-RFP	<i>ADH1</i>	Leu2	High copy	This study
pGFP-Pus1	<i>ADH1</i>	Ade2	Centromeric	Hellmuth <i>et al</i> (1998)
pH124-RFP-Pus1	<i>ADH1</i>	Leu2	Centromeric	This study
pQ103-GFP	<i>GAL1</i>	Ura3	High copy	Meriin <i>et al</i> (2002)
pRS306-GFP-ATG8	<i>ATG8</i>	Ura3	Integrative	Shintani and Klionsky (2004)
pVTG12	<i>URE2</i>	Leu2	Centromeric	Edskes <i>et al</i> (1999)
pH400tef2-Btn2-GFP	<i>TEF2</i>	Trp1	High copy	This study
pYES52-Btn2-GFP	<i>GAL1</i>	Ura3	High copy	This study
pYES52-Btn2-RFP	<i>GAL1</i>	Ura3	High copy	This study
pYES52-GST-Btn2	<i>GAL1</i>	Ura3	High copy	This study
pYES52-HOOK1 (2)	<i>GAL1</i>	Ura3	High copy	This study
pH125tef2-HOOK1 (2)	<i>TEF2</i>	Leu2	High copy	This study
pGEX-6P-GST-Btn2	<i>Lac</i>	Amp	<i>E. coli</i> origin	This study
pH376-Ure2	<i>GAL1</i>	Leu2	High copy	Bradley <i>et al</i> (2002)
yep181-Sup35NM-GFP	<i>SUP35</i>	Leu2	High copy	Ter-Avanesyan Lab

Standard rich (YPD) or synthetic (SC) yeast media were used (Sherman, 1991). Adenine-poor medium ($\frac{1}{2}$ YPD) contains half the normal amount of yeast extract and was used for the red–white assay of ADE2 expression. For nitrogen starvation, SD(–N) medium (0.17% yeast nitrogen base without ammonium sulphate and amino acids and 2% glucose) was used.

Plasmids

Plasmids used are listed in Table V. The *S. cerevisiae* genomic library (catalogue no. 37323) was purchased from American Type Culture Collection (Manassas, VA). Individual genes were tested for [URE3] curing by cloning into pRS425 yeast high-copy vector. Plasmid construction is described in Supplementary data.

Prion induction and curing

[URE3] or [*PSI*⁺] was cured with 3 mM GuHCl. To quantify [URE3] loss from overproduction of a protein, about 30 yeast colonies from a transformation plate were inoculated in liquid YPD media and grown overnight to allow plasmid loss (which was ~90% complete), and about 10⁴ cells were spread on a YPD plate. The ratio of red to white colonies was scored. Prion stability of wild-type versus *btn2Δcur1Δ* was tested under 10% DMSO treatment (overnight in liquid YPD), 0.035% HCl in SC plates (2 days growth) and 0.2 mM latrunculin A (Sigma-Aldrich, St Louis, MO; 7 h in liquid YPD). After treatment, cells were spread on $\frac{1}{2}$ YPD and the percentage of cured cells was counted.

For [URE3] induction, [ure-o] cells carrying pH376-URE2 (a full-length *URE2* coding sequence under the control of the *GAL1* promoter) were grown in SC media with 2% raffinose overnight to OD₆₀₀ = 0.5. Induction of Ure2p was started by adding galactose (2% final concentration). After 2 days of incubation, cells were counted and about 10⁶ cells were spread on plates lacking adenine to allow [URE3] selection. Ade⁺ clones were checked on 3 mM GuHCl plates to confirm the presence of [URE3].

Propagons per cell was calculated according to Cox *et al* (2003). After overnight growth on YPD, cells were subjected to 3 mM GuHCl treatment in YPD. Aliquots were periodically plated on $\frac{1}{2}$ YPD and the percentage of prion-containing colonies was plotted versus number of cell divisions. The number of propagons can be estimated from the value of 2^g, where g is the number of generations elapsed at the time point when 36.8% of cells had lost the prion. During the experiment, all cell cultures were maintained in log phase by persistent subculturing into fresh liquid YPD media with 3 mM GuHCl.

Ure2p amyloid assembly and expression of recombinant proteins

Thioflavin-T fluorescence was used to follow Ure2p amyloid formation using a method derived from previous studies (Lian *et al*, 2007). In fibrillization buffer (50 mM Tris pH 7.5 and 200 mM

NaCl), 10 μM Ure2p was mixed with 20 μM experimental proteins in a volume of 1 ml in quartz cuvettes with micro-stir bars. At time zero, 50 μl of thioflavin-T working solution (0.08 mg/ml thioflavin-T in fibrillization buffer) was added to the cuvettes and fluorescence was monitored (λ_{ex} = 420 nm; λ_{det} = 480 nm) during constant stirring at room temperature.

Proteins used in the fluorescence assay were expressed in *Escherichia coli* strain BL21-CodonPlus[®](DE3)-RIPL (Stratagene). Ure2p was prepared from the expression plasmid pKT41, as described earlier (Wickner *et al*, 2006). Btm2p was expressed as a GST fusion protein (pGEX-6P-GST-Btm2 plasmid). GST and GST-Btm2p were purified using the prescribed method for GSTrap columns (GE Healthcare). Following purification, all proteins were dialysed into fibrillization buffer and filtered (0.2 μ). Protein aliquots were frozen at –80°C before use.

Microscopy

Spinning disc confocal imaging of live yeast cells expressing the appropriate GFP- and RFP-tagged fusion proteins was performed on a Nikon Eclipse E800 microscope with a Perkin-Elmer Ultraview LC1 CSU10 scanning unit and an argon/krypton ion laser (Melles Griot, Carlsbad, CA), and an ORCA ER cooled CCD camera (Hamamatsu, Japan). Image acquisition and analysis were carried out with Openlab 3 software (Improvision, Lexington, MA). Nucleoplasmic protein Pus1p was used to confirm the nuclear localization of Cur1p. During the colocalization experiments, cells were grown overnight in 2% raffinose SC medium and then 2% galactose was added for the induction of Btm2p. Following the induction, cells were plated to $\frac{1}{2}$ YPD and the percentage of cured cells was counted. To stop cellular division, 50 μM alpha factor was added at the beginning of the induction.

Analysis of yeast cell lysates and immunoprecipitation

Strain BY241 was grown in liquid SC media to an OD₆₀₀ of 1.5. The cells were harvested, washed in buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol and complete protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) and lysed by glass beads in the same buffer. Cell debris was removed by centrifugation at 4000 g for 10 min. Protein was measured with BCA reagent (Pierce, Rockford, IL). For sedimentation analysis, cell lysates were clarified at 700 g for 10 min, then spun at 6000 g for 5 min (the pellet and the aliquot of supernatant were collected). Finally, the supernatant was placed in a new Eppendorf tube and subjected to centrifugation at 16 000 g for 10 min (the pellet and the supernatant were collected). The interaction between Btm2p–GFP and Ure2p was analysed by immunoprecipitation (IP) from cell lysates using Protein G magnetic beads (New England Biolabs, Ipswich, MA) and affinity purified antibodies against C terminus of Ure2p. Antibodies used for protein detection in western blots

were against GFP (Roche Applied Science), RFP (Rockland Immunochemicals), Hsp70/Hsc70 (no. SPA-822; Stressgen, Victoria, BC), Hsp104 (Stressgen) and Act1 (GTX80809; GeneTex, San Antonio, TX).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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