Hermes Transposon Mutagenesis Shows [URE3] Prion Pathology Prevented by a Ubiquitin-Targeting Protein: Evidence for Carbon/Nitrogen Assimilation Cross Talk and a Second Function for Ure2p in *Saccharomyces cerevisiae*

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ABSTRACT [URE3] is an amyloid-based prion of Ure2p, a regulator of nitrogen catabolism. While most "variants" of the [URE3] prion are toxic, mild variants that only slightly slow growth are more widely studied. The existence of several antiprion systems suggests that some components may be protecting cells from potential detrimental effects of mild [URE3] variants. Our extensive *Hermes* transposon mutagenesis showed that disruption of *YLR352W* dramatically slows the growth of [URE3-1] strains. Ylr352wp is an F-box protein, directing selection of substrates for ubiquitination by a "cullin"-containing E_3 ligase. For efficient ubiquitylation, cullin-dependent E_3 ubiquitin ligases must be NEDDylated, modified by a ubiquitin-related peptide called NEDD8 (Rub1p in yeast). Indeed, we find that disruption of NEDDylation-related genes *RUB1*, *ULA1*, *UBA3*, and *UBC12* is also counterselected in our screen. We find that like *ylr352w*Δ [URE3] strains, *ylr352w*Δ *ure2*Δ strains do not grow on nonfermentable carbon sources. Overexpression of Hap4p, a transcription factor stimulating expression of mitochondrial proteins, or mutation of *GLN1*, encoding glutamine synthetase, allows growth of *ylr352w*Δ [URE3] strains on glycerol media. Supplying proline as a nitrogen source shuts off the nitrogen catabolite repression (NCR) function of Ure2p, but does not slow growth of *ylr352w*Δ strains, suggesting a distinct function of Ure2p in carbon catabolism. Also, *gln1* mutations impair NCR, but actually relieve the growth defect of *ylr352w*Δ [URE3] and *ylr352w*Δ *ure2*Δ strains, again showing that loss of NCR is not producing the growth defect and suggesting that Ure2p has another function. *YLR352W* largely protects cells from the deleterious effects of otherwise mild [URE3] variants or of a *ure2* mutation (the latter a rarer event), and we name it *LUG1* (lets [URE3]/*ure2* grow).

KEYWORDS F-box protein; Gln1p; Hap4p; LUG1/YLR352W; nitrogen catabolite repression; prion; Ure2p; [URE3]

THE prions (infectious proteins) [URE3] and [PSI+] are amyloidoses of Ure2p and Sup35p, respectively, in *Saccharomyces cerevisiae* [reviewed in Liebman and Chernoff (2012), Wickner *et al.* (2015), and Saupe *et al.* (2016)], and are important models for the human prion and amyloid diseases (Kraus *et al.* 2013; Prusiner 2017). Ure2p is necessary for nitrogen catabolite repression (NCR), the shut-off of transcription of genes for the utilization of poor nitrogen sources when a good nitrogen source is available (Cooper 2002). In a [URE3] strain, most of the Ure2p is sequestered in amyloid filaments and so genes for assimilation of poor nitrogen sources (such as *DAL5*, encoding the allantoin transporter) are inappropriately derepressed, a result detected as an Ade+ phenotype in a strain with a *DAL5* promoter driving transcription of the *ADE2* gene. Sup35p is a subunit of the translation termination factor that is essential for growth (Frolova *et al.* 1994; Stansfield and Tuite 1994). [PSI+] cells have most (but not all) of their Sup35p tied up in the amyloid filaments, and therefore frequently read through termination codons.

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A single prion protein with a single amino acid sequence can form any of a large number of prion variants or strains, with different biological properties due to different conformations of the protein in the different variants (Derkatch *et al.* 1996; Collinge and Clarke 2007). Each prion variant is rather stably propagated, implying that a mechanism exists for templating of protein conformation. The parallel in-register folded β-sheet architecture known for several infectious yeast prion amyloids (Shewmaker *et al.* 2006; Baxa *et al.* 2007; Wickner *et al.* 2008; Gorkovskiy *et al.* 2014) naturally suggests a mechanism of conformational templating based on the favorable interactions of aligned identical polar or hydrophobic amino acid side chains (Wickner *et al.* 2007, 2015).

While a majority of variants of [URE3] or [PSI+] are highly toxic, or even lethal (McGlinchey *et al.* 2011), there are mild variants of each prion that are also in fact detrimental (Nakayashiki *et al.* 2005), but not severely so. Several antiprion systems have now been described in yeast, either preventing prions from arising (Chernoff *et al.* 1999) or curing most prions as they arise (Wickner *et al.* 2014, 2017; Gorkovskiy *et al.* 2017; Son and Wickner 2018). In a study of the role of the essential Hsp40 chaperone Sis1p in [PSI+] propagation, Kirkland, Reidy and Masison found that deletion of the C-terminal domain did not impair cell growth in the absence of [PSI+], but resulted in a severe growth defect on the introduction of an otherwise mild [PSI+] (Kirkland *et al.* 2011). Sis1p is necessary for [PSI+] propagation (Higurashi *et al.* 2008) and the C-terminal deletion mutants were not losing [PSI+], but were no longer protecting the cells from [PSI+] toxicity (Kirkland *et al.* 2011). We carried out a general screen for such genes that normally protect the cell from adverse effects of a prion. We used a transposon mutagenesis method based on the *Hermes* transposon originally from the house fly (Gangadharan *et al.* 2010; Guo *et al.* 2013).

Materials and Methods

The supplemental material has a detailed description of the culture conditions, induction of *Hermes* transposition, selection of colonies carrying a transposition, extraction of cellular DNA, PCR amplification and isolation of the junction points between transposon and chromosomal insertion site, next-generation sequencing of these sites, and analysis of the data by visual display and by counting insertions per open reading frame (ORF). The supplemental material also includes: "Exon Intron Counts.xlsx," giving the insertions in every yeast ORF, distinguishing exons from introns where appropriate; "Sorted Hits.xlsx," giving prominent hits sorted by functional group; "TY gag-pol Counts.xlsx," comparing insertions in the Ty retrotransposons at different locations in the genome; "Count Insertions in ORFs and Introns.txt," the Python program used for counting insertions; and "LUGsIGV-InsertDistributions.pptx," a slide show of insert distributions in each of 500 genes for which insertions were recovered more frequently in [ure-o] than in [URE3] cultures.

Data availability

The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures, and in the supplemental material. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.6205691.

Results

Transposon mutagenesis using Hermes

Hermes is a 2749-bp DNA transposon from the house fly related to the *Drosophila hobo* element and, distantly, to the *Ac* transposon of maize (Warren *et al.* 1994). *Hermes* transposes by excising itself from one site and inserting into another site in the host genome (Atkinson *et al.* 1993). Recently, *Hermes* has been adapted for use as a transposon in *S. cerevisiae* (Gangadharan *et al.* 2010) and in *Schizosaccharomyces pombe* (Guo *et al.* 2013) for genome-wide mutagenesis and insertion site analysis. We used *Hermes* transposon mutagenesis of strains with (YHE1609) and without (YHE1608) the [URE3-1] prion (Supplemental Material, Table S1), followed by a period of cell growth, PCR amplification of insertion sites from extracted DNA, and next-generation sequencing, seeking genes whose interruption by the transposon would impair growth or survival in the presence of the prion but not without it. The detailed protocol is given in *Materials and Methods* and in the Supplemental Material.

The transposon launch plasmid, pSG36, carries a *GAL*-promoted hyperactive form of the *Hermes* transposase (G366W, M286T) and a *Hermes* transposon with the *NatMX* gene (nourseothricin-resistance; NAT) on a *URA3 CEN* backbone (Gangadharan *et al.* 2010). Because *Hermes* excises from the plasmid to transpose into the chromosome, the plasmid is damaged and lost after transposition. Even when grown without uracil and without transposase synthesis (glucose), ~10% of cells lack pSG36 because the *CEN6* of pSG36 was intentionally destabilized by mutation (Gangadharan *et al.* 2010). To maintain the plasmid, the transposase induction is done without uracil. As a result, cells in which *Hermes* has transposed soon stop growing. Thus, each transposition is represented by one or a few cells (at this stage), and early transpositions are not unduly amplified. Cells lacking the plasmid were then amplified by growth with uracil and 5-FOA (5-fluoro-orotic acid) to kill Ura3+ cells, and glucose to shut off transposase synthesis and further transpositions. Finally, cells with an integrated transposon were further amplified by growth in media containing glucose, uracil, 5-FOA, and NAT. During this amplification, cells with transposon insertions in genes needed to protect cells from the prion are selectively lost in [URE3] cultures compared to [ure-o] cultures. Cellular DNA was extracted and used to PCR amplify the junctions of the integrated transposon and chromosomal DNA. An Illumina platform was used to obtain 50–130 $\times$ 10^6 reads from each sample. Methods of data analysis are detailed in the Methods section in the supplemental

Table 1 Total sequence reads and unique insertions in ORFs of the entire genome and in Ty1 transposons

	[URE3] − adenine		[ure-o] + adenine		[URE3] + adenine		[URE3] + adenine	
	Total	Unique	Total	Unique	Total	Unique	Total	Unique
Ty1 elements								
Reads	4,843,313	12,005	3,893,719	21,962	4,215,067	21,170	2,167,896	13,446
[URE3] − adenine /[ure-o] + adenine	1.24	0.547						
Amplification: growth and PCR	403.4		177.3		199.1		161.2	
All ORFs								
Reads	57,042,201	123,762	45,431,202	322,123	55,268,447	286,166	37,268,448	175,837
[URE3] − adenine /[ure-o] + adenine	1.26	0.384						
Amplification	460.9		141.0		193.1		211.9	

material. The numbers of total reads and unique insertions identified in each ORF are given in Excel file, "Exon Intron Counts.xlsx."

As a control for neutral genes, we examined the total reads and unique reads in both all ORFs and those in 42 nonessential (actually undesirable) Ty elements spread throughout the genome (Figure S2, Table 1, and supplemental file Ty gag-pol Counts.xlsx). The frequency of inserts in Ty elements varied substantially with considerably higher insertion frequencies in Ty2 elements than into Ty1, Ty3, Ty4, or Ty5 (Figure S2 and supplemental file Ty gag-pol Counts.xlsx). However, the ratios of insertions among the different strains and conditions used showed only modest variation among different Ty elements or (nearly) identical Ty elements at different locations (Figure S2). The distribution of insertions across each chromosome is quite uneven (chromosome I is shown in Figure S3), in part reflecting essential or desirable genes, and in part probably differences in chromatin access. However, the overall pattern is remarkably similar among the different cultures.

In these experiments, [URE3] is scored using an integrated *DAL5* promoter driving the *ADE2* gene. Data were obtained from [ure-o] cells grown with adenine, [URE3] cells grown without adenine, and [URE3] cells grown with adenine. As expected, cells grown without adenine could not tolerate insertions in any gene in the *ADE* pathway, independent of [URE3] (Figure S4 and supplemental file Sorted Hits.xlsx). Because the [URE3]+Ade cultures eventually accumulated a substantial fraction of [ure-o] cells (Table S2), differences with the [ure-o]+Ade culture were reduced. Nonetheless, significant differences could still be observed and these data served to distinguish the effects of [URE3] from the effects of adenine deficiency.

Even if Ure2p was inactive because it was in prion form, cells growing without adenine need an active *DAL5* promoter to drive *ADE2*. Insertions in the *GAT1* and *DAL81* genes, on which *DAL5* expression depends (Turoscy and Cooper 1982; Georis *et al.* 2008), were rare and poorly expanded in [URE3] cells growing without adenine, as expected (Figure S5 and supplemental file Sorted Hits.xlsx). Both the number of distinct insertion sites (insertion events) and their representation among the sequences (reflecting relative abundance as a result of growth and PCR amplification) were decreased compared to genes nonessential under these conditions. *GLN3* is also important for expression of *DAL5* (Turoscy and Cooper 1982; Georis *et al.* 2008) and the distribution of *Hermes* insertions showed few sites of insertion in *GLN3* in [URE3]−Ade compared to [ure-o]+Ade, as expected (Figure S5). However, an insertion at a single site in *GLN3* was massively amplified in the [URE3]−Ade sample, exceeding total runs in the [ure-o]+Ade sample. This illustrates the importance of monitoring insertion distribution (supplemental file LUGsIGV-InsertDistribution.pptx) instead of simply relying on total ORF insertion numbers. Also, many insertions were found at the 3′ end of ORFs known to be essential for growth.

The strain used also carried *CAN1*, the arginine transporter, driven by the *DAL5* promoter, so that loss of [URE3] could be selected as resistance to canavanine, a toxic arginine analog (Brachmann *et al.* 2005). As a result, arginine auxotrophs were selected against in [ure-o] strains (supplemental file Sorted Hits.xlsx).

Differences in total sequence reads in a gene can be due to differences in (1) availability for insertion (illustrated by the Ty data), (2) differences in cell growth after insertion, or (3) differences in amplification by PCR. Source 1 is assumed to be controlled by comparison of the same sites in [URE3] and [ure-o] cells, but a difference in an NCR-controlled gene might be a result of altered chromatin structure due to NCR. Source 3 is largely a problem of genes with few unique insertions in either strain. Source 2 is what we are trying to measure. All samples had roughly equal total read counts, so we have shown absolute read numbers in the tables.

Chaperone effects in [URE3] strains: Table 2 shows that insertions in a wide array of chaperone genes were markedly disadvantageous to the [URE3] strain compared with the isogenic [ure-o] host. Of course, chaperone genes necessary for [URE3] propagation, such as Hsp104 (Moriyama *et al.* 2000), Ssa2p (Roberts *et al.* 2004; Sharma and Masison 2008), Sse1p (Kryndushkin and Wickner 2007), Fes1p (Kryndushkin and Wickner 2007), Cpr7p (Kumar *et al.* 2015), and Swa2p (Troisi *et al.* 2015), were expected to appear only rarely because we selected for retention of [URE3]. Insertions in *SWA2* appeared rarely in any sample because mutants are known to grow slowly (Gall *et al.* 2000; Pishvaee *et al.* 2000). Unique insertions in *HSP104* were not unusual

Table 2 Transposon insertions in chaperone-encoding genes

Gene		Total reads			Unique inserts		
		[URE3] − adenine	[ure-o] + adenine	[URE3] + adenine	[URE3] − adenine	[ure-o] + adenine	[URE3] + adenine
Genome total in ORFs		57,042,201	45,431,202	55,692,869	303,010	710,420	627,085
HSP82	*YPL240c*	1,665	18,663	12,922	26	160	110
HSC82	*YMR186W*	335	7,899	24,898	14	121	117
HSP104	*YLL026W*	2,340	122,764	8,098,849	91	310	321
YDJ1	*YNL064c*	0	2,175	1,554	0	20	7
SWA2	*YDR320c*	0	2	8	0	2	50
CAJ1	*YER048c*	112	957	343	3	32	20
CPR7	*YJR032W*	13	224	605	4	19	21
STI1	*YOR027W*	0	1,588	3,645	0	80	74
FES1	*YBR101c*	0	344	581	0	22	17
SSE1	*YPL106c*	2,674	5,498	30,377	20	115	130
HSP42	*YDR171W*	806	14,004	24,364	21	104	95
HSP26	*YBR072W*	3,630	9,741	5,921	10	59	28
SSB1	*YDL229W*	2,007	14,463	29,150	38	182	177
SSB2	*YNL209W*	1,659	11,839	23,213	37	166	162
SSA1	*YAL005c*	176	8,562	46,571	11	219	203
SSA2	*YLL024c*	287	6,775	36,604	12	147	126
SBA1	*YKL117W*	559	1,800	1,846	7	35	24

in the [URE3] strain, but they were not amplified compared to the [ure-o] host because they eventually lost [URE3] and became Ade−. In cultures with added adenine, cells with insertions in *HSP104* became abundant because they lacked the growth-slowing [URE3] prion (Wickner 1994; Schwimmer and Masison 2002).

However, many other chaperone genes, including those known not to be necessary for [URE3] propagation, were underrepresented in the collection of insertions. The duplicated Hsp90-encoding genes *HSP82* and *HSC82*, and the duplicated *SSB1* and *SSB2*, were all underrepresented in the [URE3] compared to the [ure-o] condition (Table 2). It is possible that the requirement for chaperones to deal with the stress of [URE3] prion infection, combined with a chaperone deficiency, gives a synthetic slow growth resulting in underrepresentation of this group in our competitive growth environment.

Particularly striking is the fact that no insertions in *STI1* were recovered in the presence of [URE3] (Table 2). Sti1p is a cochaperone for Hsp90s (Hsp82 and Hsc82 in yeast), Hsp104, and Hsp70s (Scheufler *et al.* 2000; Abbas-Terki *et al.* 2001), which has already shown several antiprion activities. Overproduction of Sti1p cures some [PSI+] variants (Kryndushkin *et al.* 2002) and promotes loss of [PSI+] in an *ssa1* mutant (Jones *et al.* 2004), although [URE3] is not cured (Lian *et al.* 2007). Sti1p is also necessary for the curing of [PSI+] by overproduction of Hsp104 (Moosavi *et al.* 2010; Reidy and Masison 2010) and for the curing of many [PSI+] variants by normal levels of Hsp104 (Gorkovskiy *et al.* 2017). Sti1p also protects cells from the toxicity produced in [PIN+] cells by the overproduction of Rnq1p or of Htt103Q, an expanded toxic version of Huntingtin (Wolfe *et al.* 2013).

Insertion in URE2 is rare in [ure-o] and common in the [URE3] host: It may seem paradoxical that insertions in *URE2* were far more common in the [URE3] strain (with or without adenine) than in the [ure-o] host, because in both cases, Ure2p is largely or completely inactivated (supplemental file Sorted Hits.xlsx). However, most [URE3] cells grow slower because of the prion than they would simply by losing Ure2p because of *ure2*Δ (McGlinchey *et al.* 2011). Deletion (*ure2*Δ) relieves the prion-induced growth inhibition and cells grow better than all the other ([URE3]) cells. In wild-type cells, *ure2*Δ makes cells grow slightly slower than normal, and so insertions in *URE2* are counterselected. Essentially, the results follow from the fact (McGlinchey *et al.* 2011) that [URE3] is usually worse for cells than is *ure2*Δ.

YLR352W/LUG1 (lets [URE3] grow): The strongest signal for a gene that improves fitness dramatically in a [URE3] strain was *YLR352W* (Figure 1B and Table 3). In a [ure-o] host, 127 distinct insertions (on average one per 19 bp) were found in *YLR352W*, which amplified to nearly 10,000 reads by cell growth and PCR. While PCR inevitably amplifies different fragments somewhat differently, it is likely that such differences even out over an entire ORF. In a [URE3] host grown without adenine, only one insertion, unamplified, was observed, suggesting a difference in amplification by cell growth after the transpositions into *YLR352W* and the selective loss of many insertions in this gene. When the transposition was induced in cells carrying [URE3] in the presence of adenine, there was still a substantial reduction of insertions and little amplification of those that did occur (Figure 1 and Table 3). However, the reduction was less dramatic in this case, perhaps because, in the absence of selection for the prion, a substantial fraction of [ure-o] cells accumulated (Table S2, C and D).

SCF ubiquitin ligases are protein complexes consisting of Skp1p, a cullin [a structural framework for the complex; in

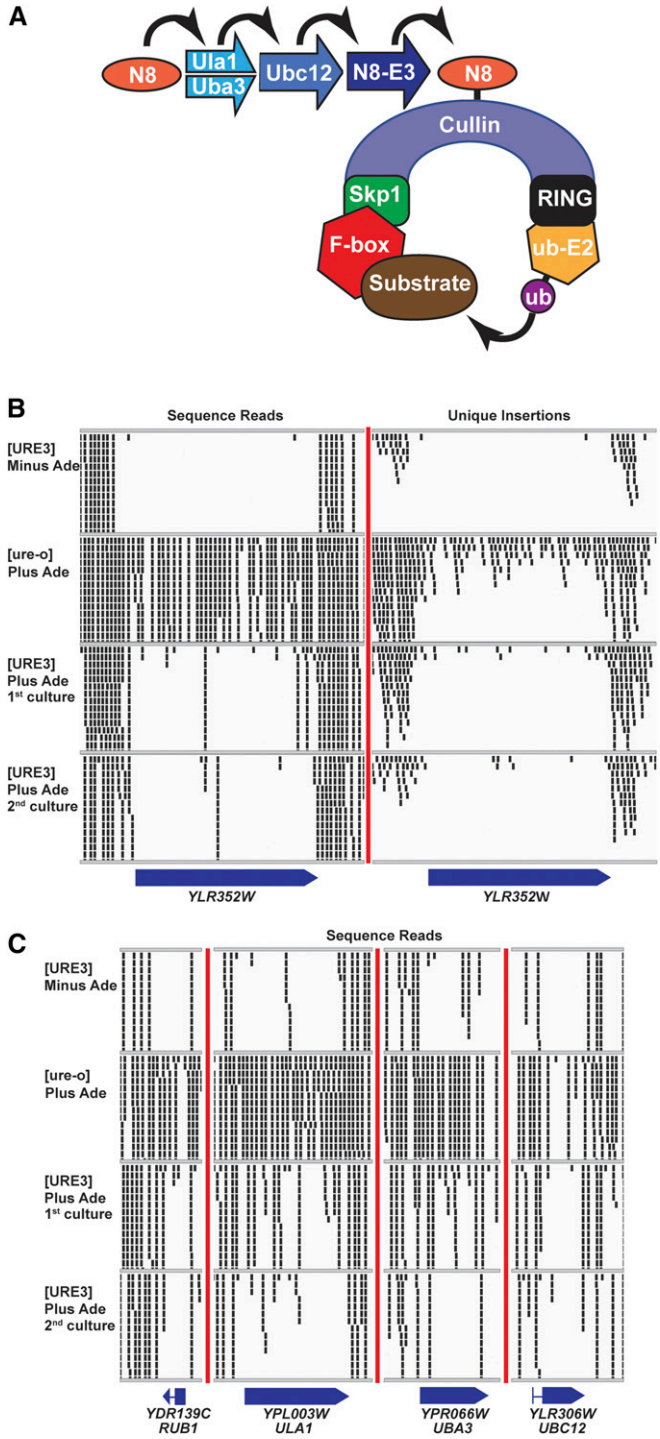

Figure 1 (A) The SCF complex ubiquitination system. Ylr352wp/Lug1p is one of 20 yeast F-box proteins specifying substrates for ubiquitination by SCF complexes (see text). Yeast cullins include Rtt101p, Cdc53p, Cul3p, and Apc2p. The RING protein is Hrt1p. The E2-ubiquitin ligase is Cdc34p. NEDD8 (Rub1p in yeast; N8 in the figure) is a ubiquitin-like peptide whose attachment and removal from cullin is required for cycling of the SCF complex. (B) Insertions in *YLR352W/LUG1* are recovered less frequently in [URE3] cells compared to [ure-o] cells. Although there were more total sequence reads for the [URE3]−Ade or [URE3]+Ade first cultures than for the [ure-o] culture, few were recovered in *YLR352W/LUG1*. The small rectangles represent the 50 bp to the right of the *Hermes* integration site. Only a maximum of 14 reads at one site, including overlapping sites, are shown. The totals in the tables include all reads/insertions. This figure is produced using IGV (http://software.broadinstitute.org/software/igv/). (C) NEDDylation genes are important for the ubiquitinylation process by SCF complexes and their mutations are also selectively underrepresented in [URE3] cultures. Total sequence reads are shown. Unique insertions are shown in Figure S5.

yeast Cdc53, RTT101, or CUL3 (Sarikas *et al.* 2011)], Cdc34p (the catalytic subunit), Hrt1 (RING protein, connects Cdc34p to Cdc53), and one of 20 F-box proteins (substrate-specifying subunit bound by the F-box sequence to Skp1p) (Jonkers and Rep 2009; Hua and Vierstra 2011) (Figure 1A). Lug1p/Ylr352wp is one such F-box protein, which has been shown to bind to Skp1p (by two-hybrid experiments) and to Cdc53p (by affinity purification), but whose target proteins are not known (Seol *et al.* 2001).

NEDD8 is a ubiquitin-like (60% identity) peptide that modifies a limited range of proteins, primarily the cullin subunits of SCF ubiquitin ligases (Enchev *et al.* 2015). This NEDDylation of cullins enhances the activity of SCF ubiquitin ligases by allowing the exchange of F-box proteins (Figure 1A). If Lug1p is needed for growth of [URE3] strains because of its role in SCF complex ubiquitin ligation, then the NEDDylation genes should also be necessary. The yeast NEDD8 is encoded by *RUB1*, and Ula1p and Uba3p form a complex that acts for NEDDylation like an E1 enzyme for ubiquitin. *UBC12* encodes the E2 analog (Lammer *et al.* 1998; Liakopoulos *et al.* 1998). Indeed, a clear deficiency of reads was seen in *RUB1*, *ULA1*, *UBA3*, and *UBC12* in [URE3] cells compared to [ure-o] cells (Figure 1C, Figure S6, and Table 3). Lag2p binds to cullins and inhibits NEDDylation (Liu *et al.* 2009), and Yuh1p is responsible for removal of NEDD (Rub1p) (Linghu *et al.* 2002), and both were significantly less often mutated by *Hermes* in the [URE3] strain, consistent with their roles in the NEDDylation cycle (Table 3).

Growth defect of lug1Δ [URE3-1] and NEDDylation defective [URE3] strains: To confirm the apparent poor fitness of the normally mild prion variant [URE3-1] in strains lacking either the F-box protein Lug1p or one of the NEDDylation components Rub1p, Ula1p, Uba3p, or Ubc12p, we transferred cytoplasm (cytoduced) from [URE3-1] or [ure-o] donors into recipients deleted for one of each of the genes encoding these factors (Figure 2). Recipient cells were made ρ^o by growth on ethidium [so that growth on glycerol medium (YPG) could be used as an indication of receipt of donor cytoplasm] and carried a recessive *cyh2* allele, making them cycloheximide-resistant. Cytoductants were selected on YPG containing cycloheximide. Note that diploids would be cycloheximide-sensitive because the *cyh2* allele in the recipient is recessive. Unmated recipients will not grow on YPG because they are ρ^o. When [URE3] was introduced into the *lug1*Δ strain, no growth was seen, although cytoplasm from the same donor without the prion did not substantially affect growth (Figure 2). This result explains and confirms the outcome of the *Hermes* transposition experiment. Each deletion

Table 3 Transposon insertions rare in *YLR352W/LUG1* and NEDDylation-related genes

Gene		Total reads			Unique inserts		
		[URE3] − adenine	[ure-o] + adenine	[URE3] + adenine	[URE3] − adenine	[ure-o] + adenine	[URE3] + adenine
Genome total in ORFs		57,042,201	45,431,202	55,692,869	303,010	710,420	627,085
F-box protein							
LUG1	*YLR352W*	1	9,882	344	1	127	17
NEDDylation							
RUB1	*YDR139C*	0	574	24	0	13	4
ULA1	*YPL003W*	43	8,200	1,513	7	112	31
UBA3	*YPR066W*	30	9,874	307	5	69	24
UBC12	*YLR306W*	21	8,458	9,266	2	31	12
DCN1	*YLR128W*	392	1,301	618	4	23	14
Cullins							
RTT101	*YJL047C*	488	3,358	1,511	14	61	71
CDC53	*YDL132W*	8	571	242	1	6	7
APC2	*YLR127C*	417	3,360	1,601	5	20	11
deNEDDylation							
LAG2	YOL025W	1143	3,622	1,934	14	59	53
YUH1	YJR099W	520	5,145	838	9	31	22

Among the 20 yeast F-box protein genes, only *YLR352W/LUG1* shows a dramatic deficit of insertions in the [URE3] host. The cullin gene *RTT101* also showed only rare mutation in the [URE3] host without adenine. DeNEDDylation genes show a modest decrease in the [URE3] host. Frequent insertions in the [URE3] cells grown with adenine may be due to accumulation of [ure-o] cells in these cultures (Table S2, C and D).

mutant in the NEDDylation genes could grow on the YPG + cycloheximide medium after receiving [URE3], except for *uba3*Δ (Figure 2), which is known to be respiratory incompetent independent of [URE3] (Merz and Westermann 2009). The receipt of [URE3] by these cytoductants, including the very slow-growing *uba3*Δ [URE3] and *uba3*Δ [ure-o] cytoductants, was confirmed by their ability to grow on –Ade plates and their white color on adenine-limiting (1/2 YPD) medium (Figure S7).

Meiotic analysis confirmed that *lug1*Δ strains grew poorly on glycerol medium, specifically if [URE3] was present (Figure 3). The *lug1*Δ [ure-o] strain YHE1633 was crossed with the *LUG1* [URE3-1] strain YHE1627, the diploids were sporulated, and tetrads dissected and germinated on YPAD (rich dextrose medium = yeast extract peptone adenine dextrose). Spore clones with the *lug1*Δ disruption and [URE3] were viable, but were slow growing on rich dextrose medium and did not grow on rich glycerol medium (YPG) (Figure 3). In contrast, *lug1*Δ [ure-o] segregants (red on 1/2 YPD) grew well on YPAD or YPG [note that [URE3-1] × [ure-o] meiotic crosses are known to produce some [ure-o] segregants (Lacroute 1971)]. To test whether mitochondrial DNA was lost or damaged in the *lug1*Δ [URE3] segregants, all segregants were mated with *LUG1* ρ° strains and the diploids tested for growth on YPG. The diploids in each case could grow on YPG (Figure 3), showing that the mitochondrial DNA was intact. Also, mitochondria had a normal appearance when visualized with mitoDsRed (Figure S8).

Cytoductants for the NEDDylation mutants and *lug1*Δ [URE3] meiotic segregants (see above) were also tested for growth on dextrose or galactose–raffinose, the two carbon sources used in the transposon experiment (Figure 4 and Figure 5). The *lug1*Δ and each of the NEDDylation-related deletion strains grew well on dextrose medium if [ure-o], but poorly if [URE3]. The same was true for galactose–raffinose medium, except that again the *uba3*Δ strain grew poorly even without [URE3] (Figure 4).

Growth defect of lug1Δ ure2Δ strains: The growth problem of *lug1*Δ [URE3] could be due to the presence of the amyloid filaments of Ure2p or due to the absence of Ure2p activity. To differentiate between these two possibilities, *ure2*Δ cells were mated with *lug1*Δ cells lacking [URE3] and, after sporulation, tetrads were analyzed. Spore clones that contained both *ure2*Δ and *lug1*Δ were viable, but were slow growing on dextrose-rich medium (YPAD) and did not grow on YPG

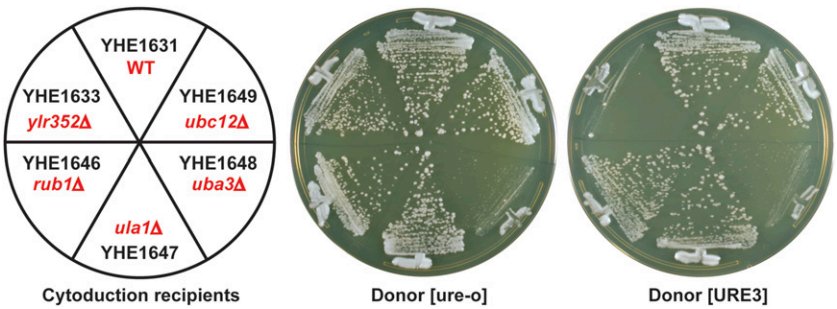

Figure 2 Cytoduction (cytoplasmic transfer) from the [URE3] strain YHE1627 or the [ure-o] strain YHE1635 into wild-type and deletion strains was carried out. Cytoductants, carrying the nucleus of the recipients and a mixture of donor and recipient cytoplasm, were selected on rich glycerol medium (YPG) containing 3 μg/ml cycloheximide, and photographed after 7 days at 30°.

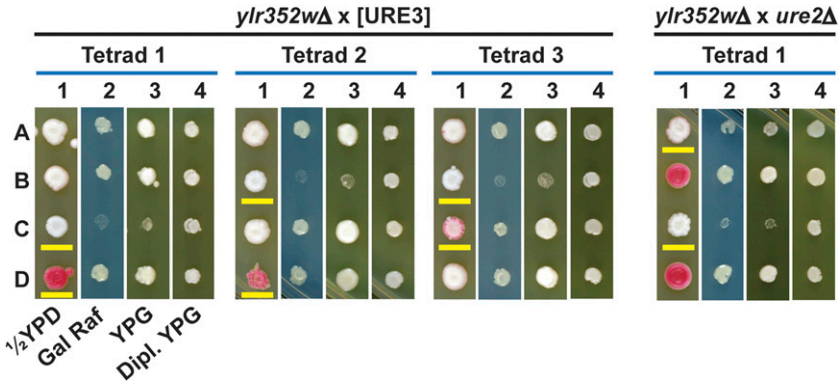

Figure 3 Meiotic crosses of *lug1*Δ strains with [URE3] and *ure2*Δ strains. Left: *lug1*Δ strain YHE1633 crossed with [URE3] strain YHE1627 showing three typical tetrads. Medium 1 = 1/2 YPD (limiting adenine), medium 2 = yeast nitrogen base medium with galactose (Gal) and raffinose (Raf) as carbon sources (2% each), medium 3 = YPG, and medium 4 = YPG growth of diploids (Dipl.) formed with the meiotic segregant and a ρ^o strain of opposite mating type as a check on loss of mitochondrial DNA. Spore clones with the *lug1*Δ allele are underlined in yellow. The occasional spore clone that spontaneously lost [URE3] appears red on 1/2 YPD. In general, *lug1*Δ [URE3] segregants could not grow on YPG and grew poorly on the minimal Gal/Raf medium. Right: A typical tetrad from a cross of *lug1*Δ strain YHE1630 and *ure2*Δ strain YHE1636. The media are as for the left cross. Segregants with *lug1*Δ are underlined in yellow and *ure2*Δ segregants appear white on 1/2 YPD (medium 1). In general, *lug1*Δ *ure2*Δ segregants did not grow on YPG and poorly on minimal Gal/Raf medium, and had not lost their mitochondrial DNA.

(glycerol-rich medium) (Figure 3). Analysis of 34 tetrads showed that nearly all *lug1*Δ *ure2*Δ spore clones grow poorly on YPG and that nearly all subclones growing on YPG had a *gln1* suppressor mutation (see below). Thus, it is the absence of Ure2p rather than the presence of Ure2p amyloid that causes the observed phenotypes. Expression in the *lug1*Δ *ure2*Δ cells of full-length Ure2p or the C-terminal portion responsible for NCR corrects the growth defect, but expression of the N-terminal prion domain does not (Figure S9).

Inactivation of Ure2p on a proline nitrogen source does not slow growth of lug1Δ strains: When yeast has a good nitrogen source such as ammonia (present in Yeast Nitrogen Base), it shuts off the transcription of genes for the utilization of poor nitrogen sources, a process called NCR and requiring an active Ure2p. On a poor nitrogen source, such as proline, this repressing activity of Ure2p is inactivated. If it is this NCR-mediating activity of Ure2p that is needed for growth in a *lug1*Δ strain, then *URE2 lug1*Δ cells should grow poorly on medium whose nitrogen source is proline. We find that *lug1*Δ cells grow as well as wild-type cells on either proline–glucose medium or on ammonia–glucose medium (Figure 5). Thus, the functional inactivation of Ure2p that occurs when NCR is shut off does not produce the toxicity seen for a *ure2*Δ strain or a [URE3] strain.

High copy HAP4 suppresses lug1 [URE3] growth defect: To obtain some insight into the growth defect in *lug1*Δ [URE3] cells, we introduced a high-copy library (Jones *et al.* 2008) into the *lug1*Δ *cyh2*(Q38K) [ure-o] strain YHE1633 (*MAT***a** *ura3 leu2 trp1 kar1* [ure-o] *lug1*::*kanMX* cyh^R rho^0). We looked for cells, deleted for *LUG1*, that would tolerate [URE3] in the presence of a high-copy plasmid containing parts of the yeast genome. Plates with ~200 transformants each were replica plated onto a lawn of [URE3] donor strain YHE1627 (*MAT*α *ura3 leu2 his3*::*TRP1*^{alb} *trp1 kar1* [URE3-1] rho^+). This allows cells to mate and, due to the *kar1* mutation, cytoductants to form. Cytoductants will contain the nuclear information of the *lug1*Δparent but have the cytoplasmic contents of both parents. After overnight incubation at 30°, the mating plate was replica plated onto YPG medium containing 3 μg/ml cycloheximide. This selects for *lug1*Δ deletion cells that have obtained mitochondria, and thus also [URE3], and are capable of growing on glycerol medium due to the presence of a plasmid from the library. In total, we screened 11,800 colonies covering the library of 1588 plasmids around seven times.

In addition to a plasmid carrying *LUG1* and plasmids curing [URE3] because they overexpress Sse1p, Ydj1p, or Btn2p [see Moriyama *et al.* (2000), Kryndushkin and Wickner (2007), Kryndushkin *et al.* (2008)], the screen yielded the overlapping plasmids YGPM17p16 (four times) and YGPM3b23 (once). The overlap region of these plasmids contains two genes: *HAP4*, encoding a transcriptional activator of many mitochondrial genes on respiratory substrates (Lascaris *et al.* 2002), and *SLD2*, encoding a single-stranded DNA origin-binding and annealing protein. Overexpression of just the *HAP4* gene was then found to be sufficient to suppress the growth defect of *lug1*::*kanMX* [URE3] strains (Figure S10). The suppression of the growth defect on glycerol by Hap4p overproduction indicates that the growth defect is in some aspect of mitochondrial carbon utilization.

Spontaneous gln1 mutations suppress the growth defect of lug1Δ [URE3] strains: Strain YHE1674 (*lug1*::*kanMX* [URE3-1]) does not grow on YPG, but 36 rare YPG+ clones were isolated. Of these, eight clones had lost [URE3] (Ade−, red on YES medium), explaining their restored growth on YPG. Thirteen of the remaining clones were tested for the presence of [URE3] by cytoduction into a wild-type [ure-o] (YHE1714) recipient, and each transmitted [URE3] efficiently. As before, transmission to a *lug1*Δ recipient again produced failure of recipient growth on YPG, indicating that the [URE3] in the donors had not changed in this respect. After growth of each of these strains on rich medium with 3 or 5 mM guanidine to cure [URE3] [by inhibition of Hsp104 (Jung *et al.* 2002)], they were cytoduced into the same wild-type recipient (YHE1714) and none transmitted

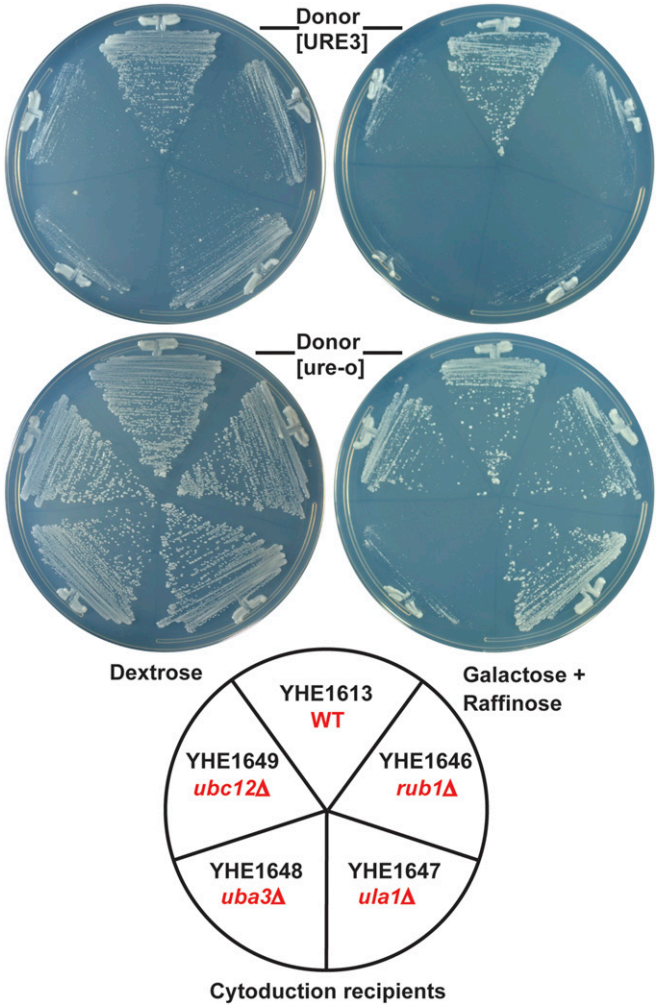

Figure 4 Test of incompatibility of [URE3] and NEDDylation gene mutants *rub1*, *uba3*, *ula1*, and *ubc12*. Cytoductants of [URE3] (or the [ure-o] control) into knockouts of *rub1*, *uba3*, *ula1*, and *ubc12* from Figure 2 were tested for growth on media with either dextrose or galactose/raffinose as a carbon source (2% each). Plates were minimal medium supplemented with Ura, Leu, Trp, and Ade, and incubation was at 30° for 2 days for dextrose plates and 3 days for galactose plates. WT, wild-type.

[URE3]. However, although [URE3] had evidently been cured, the strains that had been grown in the presence of guanidine remained Ade+ and white on adenine-limiting plates (Figure S11). A *ure2* mutation could explain this phenomenon, but sequences of six of these clones showed normal sequence of *URE2*, including 280 bp upstream and 368 bp downstream of the ORF.

All 13 *lug1*Δ [URE3] YPG+ clones tested, when crossed with the *lug1*Δ strain YHE1721, produced diploids that grew poorly on YPG and, once cured of [URE3], became red on limiting adenine, implying that the mutation conferring growth on YPG and white color in spite of being [ure-o] is recessive. To find the affected gene, we transformed one of the YPG+ clones (YHE1760) cured of [URE3] (but still white, see above) with the same yeast genomic library (Jones *et al.* 2008) used above, looking for clones that made the cells turn red on limiting adenine. Plasmid-containing clones were selected on minimal dextrose medium containing 0.0006% adenine sulfate. This amount of adenine is sufficient to allow growth, but also allows red pigment development in Ade2-deficient cells. As explained above, in our strains, *ADE2* is controlled by the NCR system. Among almost 40,000 colonies, we isolated the *URE2*-containing plasmid YGPM26i16 five times, and plasmids with the ammonia permease genes *MEP1* (17 times) and *MEP3* (23 times). Seven isolates carried YGPM23i14, which includes *GLN1* encoding glutamine synthetase. We sequenced the *GLN1* ORF from the same six strains whose *URE2* gene is normal and found that all six had mutations in *GLN1*. YHE1760, as well as four others of these strains, had C896A, resulting in a threonine to asparagine change in the conserved amino acid residue 299. The sixth strain had G304T (D102Y) and A517G (I13V) mutations. The importance of *GLN1* in this phenomenon was confirmed by isolation of YPG+ derivatives of five *ure2*Δ *lug1*Δ strains, four of which had mutations in *GLN1*, including three different mutant alleles (G263Δ, H250Q, and double mutant T345K M348I). Based on the fact that nearly all YPG+ clones were mutant in *GLN1* and that the YPG+ and Ade+ phenotypes (even after curing [URE3]) coincided in all mutants, the complementation of both phenotypes by *GLN1* implies that the *gln1* mutations were the cause of both phenotypes.

Glutamine is a signal of nitrogen source sufficiency in yeast (Cooper 1982; Stanbrough *et al.* 1995; Crespo *et al.* 2002; Magasanik and Kaiser 2002; Stracka *et al.* 2014; Fayyad-Kazan *et al.* 2016). The fact that *gln1* mutations overcome the YPG-negative phenotype of the *lug1*Δ *ure2*Δ strains suggests that from a point in the NCR pathway at or after glutamine, but before Ure2p, a signal is sent to the carbon-assimilation control systems.

Other genes whose mutation is rarely recovered in [URE3] cells: No insertions in *TKL1*, encoding transketolase, were recovered in the [URE3] host, while a substantial number were found in the [ure-o] strain, and meiotic analysis confirmed a negative genetic interaction between *ure2*::*URA3* and *tkl1*::*kanMX*. In addition, a large number of genes, representing many functional groups were significantly less often recovered in the [URE3] host (supplemental file Sorted Hits.xlsx). Further work will be required to determine the significance of these results.

Discussion

We have adopted the house fly transposon *Hermes* system, developed by Gangadharan *et al.* in *S. cerevisiae* (Gangadharan *et al.* 2010) and used in *S. pombe* (Guo *et al.* 2013), to search for genes that become more important when a cell becomes infected with a prion. This method is particularly sensitive because the competitive growth phase allows detection of all degrees of relative fitness of the many mutants generated, as well as, of course, addressing the entire

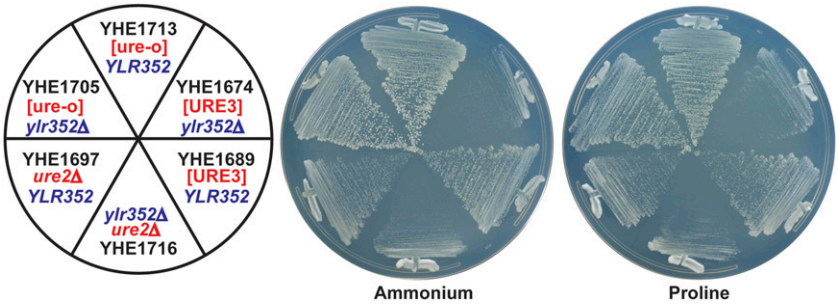

Figure 5 Inactivation of Ure2p by using proline as a nitrogen source does not make *lug1*Δ strains slow growing. Although *lug1*Δ [URE3] and *lug1*Δ *ure2*Δ cells grow poorly on minimal proline glucose plates (or minimal ammonium glucose plates), *lug1*Δ *URE2*$^+$ cells do not show slow growth on proline, although this condition is well known to inactivate the nitrogen catabolite repression activity of Ure2p (Cooper 2002).

genome at once. The utility of transposon mutagenesis and a method to use the maize *Ac* transposon for such work has recently appeared (Michel *et al.* 2017). As with transposon mutagenesis in general, different cultures accumulate insertions at a distinct distribution of sites. Thus, only those genes with a substantial number of independent insertions in at least one of the samples can be compared. Very small genes or genes in cold spots for *Hermes* integration are not well tested. In addition, there are many possible reasons why a gene may appear to be often mutated in [ure-o] strains and only rarely in [URE3] cells. The *DAL5:ADE2* and *DAL5:CAN1* constructs used to assay [URE3] differentially affect the recovery of insertions in *ADE* and *ARG* genes, as well as the NCR genes *GAT1*, *GLN3*, and *DAL81*. These results serve as controls showing that the method works. Since the insertions in one culture are not actually the same as those in another, differences in the efficiency of PCR amplification can affect the results. Even essentially identical sequences (Ty1) are differentially targeted by *Hermes* depending on their location in the genome. There are evidently differences in the accessibility of regions of the genome (Guo *et al.* 2013). However, when compared across cultures, the differences with location were consistent (Figure S2). Strains with differing chromatin structure (presumably NCR-sensitive genes in this case) could produce deceptive results, but no NCR-sensitive genes turned up in our screen.

Comparison of genes whose mutation is rarely recovered in [URE3] cells with known negative genetic interactors with ure2Δ

The *Saccharomyces* Genome Database lists 187 genes whose mutation shows negative genetic interaction with *ure2*Δ in mass screens (summarized at https://www.yeastgenome.org/locus/S000005173/interaction#annotations). Among these, 36 were detected in our screen as showing diminished frequency of mutant appearance in the [URE3] host, including *lug1* and *tkl1* (Costanzo *et al.* 2010) (Table S4). As expected, many of the known negative interactors with *ure2*Δ detected in our screen were transcription factors, or genes affecting histone modification or chromatin assembly. However, another substantial group involved the ubiquitin/proteasome system. Notably, none of the many chaperone genes detected in our screen with [URE3] had been identified in the *ure2*Δ negative interaction screens, suggesting that their role is protecting against [URE3] prion toxicity, rather than the deficiency of active Ure2p, but further work will be needed to establish this conclusion.

Our screen for genes protecting [URE3] cells from a growth defect revealed that *lug1*Δ [URE3] cells could not grow on glycerol medium and grew slowly on other carbon sources. The same growth defect is seen for *lug1*Δ *ure2*Δ cells, but [URE3] arises far more frequently than do *ure2* mutations (79 of 93 spontaneous Ade+ mutants of YHE1608 were [URE3]), so we infer that Lug1p is mostly protecting cells from the adverse effects of [URE3]. Lug1p encodes an F-box protein, a substrate-specifying subunit of the Skp1-Cullin-Cdc34- Hrt1 E3 ubiquitin ligase.

Why do lug1 [URE3] and lug1 ure2 strains not grow on glycerol?

Lug1p is an F-box protein presumed to direct the degradation of some other protein. Ure2p binding to Gln3p (and Gat1p) mediates NCR when glutamine (made by Gln1p or glutamine in the medium) is in full supply. The *lug1*Δ [URE3] and *lug1*Δ *ure2*Δ strains can grow on glycerol if either Hap4p is overexpressed or *Gln1p* is defective. Utilization of glycerol requires oxidation by mitochondria and Hap4p is a major transcription factor promoting the expression of a myriad of mitochondrial proteins. It is possible that the lack of Lug1p and Ure2p results in an inadequate supply of some mitochondrial components needed for glycerol utilization. A *gln1* mutant, presumably by not supplying the nitrogen sufficiency signal glutamine, allows the *lug1* [URE3] and *lug1*Δ *ure2*Δ strains to grow on glycerol. Glutamine seems to inhibit glycerol utilization when Ure2p and Lug1p are absent.

Does Ure2p have a function independent of NCR?

The NCR pathway is: extracellular NH_3 → intracellular NH_3 → Gln1p makes glutamine → Ure2p binds Gln3p and Gat1p → reduced transcription of genes for using poor nitrogen sources. Like [URE3] or a *ure2*Δ mutation, a *gln1* mutation or proline as the nitrogen source results in derepression of nitrogen catabolism genes. The following observations argue that Ure2p has a newly discovered role affecting carbon utilization, in addition to its well-known role in regulating nitrogen catabolism. (1) *lug1*Δ [URE3] or *lug1*Δ *ure2*Δ strains fail to grow on nonfermentable carbon sources. (2) The fact that *gln1* mutations suppress the growth defect on YPG of *lug1*Δ [URE3] and *lug1*Δ *ure2*Δ strains implies that it is not the derepressed nitrogen catabolism of these strains that is

producing the growth defect, and points to an alternate function of Ure2p. (3) We find that growth of *lug1*Δ strains on proline, which inactivates Ure2p for NCR, does not produce the growth-slowing effects that result from [URE3] or a *ure2* mutation. This result again argues that there is a distinct function of Ure2p that is independent of the NCR pathway.

While our results suggest some cross talk between the NCR and carbon pathways, we lack a clear image of the mechanism. What is the target of Lug1p that mediates these effects? What mitochondrial factor(s) deficiency is (are) making these cells unable to use glycerol? What component does glutamine act on to inhibit glycerol utilization and would that target be degraded if Lug1p were present? We have succeeded in finding a cell component, the F-box protein Lug1p, that mitigates the adverse consequences of the [URE3] prion, but further studies will be needed to understand the mechanism of this effect.

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