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Cellular tolerance of prion protein PrP in yeast involves proteolysis and the unfolded protein response

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Abstract

Secretory proteins undergo a stringent quality control process in the endoplasmic reticulum (ER). Misfolded ER proteins are returned to the cytosol and destroyed by the proteasome. Prion protein PrP is degraded by the proteasome in mammalian cells. However, the significance of proteolysis on PrP-induced cell death is controversial. Moreover, the specific pathway involved in PrP degradation remains unknown. Here, we demonstrate that the unglycosylated form of human PrP is subjected to the ER-associated protein degradation (ERAD) process in the yeast *Saccharomyces cerevisiae*. We also show that unglycosylated PrP is degraded by the Hrd1–Hrd3 pathway. Accumulation of misfolded proteins triggers the unfolded protein response (UPR), which promotes substrate refolding. Interestingly, we find that the expression of PrP leads to growth impairment in cells deficient in UPR and ERAD. These findings raise the possibility that decreased UPR activity and proteolysis may contribute to the pathogenesis of some prion-related diseases.

Keywords: Ubiquitin; ERAD; PrP; UPR; Proteasome

The majority of selective proteolysis in eukaryotes is handled by the proteasome. Substrates of the proteasome are often first covalently modified by the ubiquitin (Ub) molecule [1]. Ub, an abundant 76-residue protein, is highly conserved from yeast to human. Ub is activated and transferred to the substrate via several enzymes including a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub-protein ligase (E3). The rate-limiting step is likely the recognition of the substrate by the E3 enzyme. The ubiquitylated substrate is then degraded by proteasome. Defects in the Ub/proteasome system can lead to cancers and neurodegenerative diseases [2].

One interesting substrate of the Ub/proteasome system is human prion protein PrP. PrP is a causative agent in transmissible spongiform encephalopathy (TSE), including Creutzfeldt–Jacob disease and mad cow disease [3,4]. Prion diseases are enigmatic because of the transmissible nature by breaking the species barrier and the epigenetic nature of the infection. The precise physiological function of PrP remains elusive. In its infectious form, PrP takes on a non-native conformation from a predominately α-helical structure to a mostly β-sheet form. This altered conformation causes the protein to be prone to aggregation. Prion aggregates can convert the normal cellular protein to the aggregate form (PrP^{Sc}). The acquisition of PrP^{Sc} leads to neuropathological defects and ultimately death. Thus, the amount and activity of PrP must be tightly regulated.

Mature PrP is a glycoprotein localized at the cell surface [5]. PrP can be glycosylated at two sites N181 and N197 (Fig. 1A), although their significance remains to be elucidated. Wild-type PrP and two pathogenic PrP mutants are degraded by the proteasome [6–8]. PrP is routed through the ER to the plasma membrane to fulfill its normal, albeit unknown, function. The folding state of secretory proteins is monitored in the ER by a quality control mechanism. Immature proteins are retained to fold properly by ER chaperones. Proteins that cannot fold are disposed of via a process termed ER-associated protein

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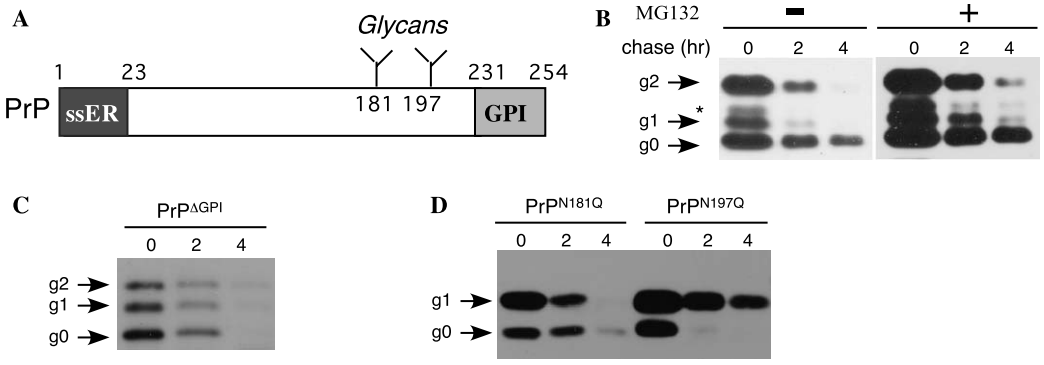

Fig. 1. Degradation of PrP and its derivatives in yeast. (A) The structural organization of PrP. Three features of PrP are highlighted: (1) an ER targeting signal sequence (ssER); all PrP alleles described here contain a yeast ER signal sequence instead of its original signal sequence to facilitate entry to the yeast ER; (2) the glycophosphatidylinositol (GPI) anchoring sequence that is removed for the attachment of the GPI moiety, which anchors mature PrP to the plasma membrane [5]; (3) two glycosylation sites, N181 and N197. (B) All forms of human PrP are degraded in yeast. Wild-type yeast cells expressing PrP were treated with or without the proteasome inhibitor MG132. Time points were taken after expression shut-off. Samples were processed for Western blotting with anti-PrP antibody (3F4). PrP stability in yeast is determined as described in Materials and methods. The glycosylation pattern was determined by Endo H treatment, indicated on the left. Arrows on the left indicate PrP linked with two (g2), one (g1), or no (g0) glycans. Asterisk denotes untranslocated PrP with its ER signal sequence attached. (C) $PrP^{\Delta GPI}$ is degraded in yeast. The stability of $PrP^{\Delta GPI}$ in wild-type cells was determined by promoter shut-off assay described as in (B). Equal sample loading was confirmed by blotting the extracts with anti-actin antibody. The identity of various glycoforms of PrP is indicated on the left. (D) Mono-glycosylated PrPs are degraded in yeast. One of the glycosylation sites was mutated from N to Q. The stability of these mutants were determined as described in (B).

degradation (ERAD) to prevent toxification by the accumulation of aberrant proteins [9,10]. More specifically, misfolded proteins are returned to the cytosol and subsequently degraded by the proteasome. Although PrP has been shown to be a substrate of ERAD, it is unclear which (glyco)form(s) of PrP is recognized as misfolded and targeted for ERAD. Inhibition of the proteasome led to the accumulation of soluble PrP in the cytosol, which was thought to be extremely toxic and promote PrP^{Sc} formation [11]. However, the toxicity may be caused by other proteins that accumulate upon proteasome inhibition. Several groups report conflicting results and suggest that PrP accumulation per se may not be sufficient to trigger cell death [12–15]. Hence, the exact function of the proteasome in prion pathogenesis remains unclear. Importantly, the specific pathway involved in PrP degradation has not been identified.

To define the role of proteolysis in prion biology, we have employed the yeast *Saccharomyces cerevisiae* as a model system, which offers powerful genetic and biochemical tools. Although yeast cells do not develop neurodegenerative disorders, studies using mammalian proteins involved in neurodegeneration (α-synuclein, Huntingtin, and also PrP) demonstrate the utility of yeast for understanding the regulation of protein aggregation, amyloid formation, and cell death [16–20]. In addition, the protein quality control process is highly conserved from yeast to human. Studies of the degradation of human proteins (e.g., CFTR) in yeast have revealed key insights, to their regulation [21]. While several groups have expressed human PrP in yeast, they focused on PrP-induced aggregation but not PrP turnover [16,18]. Here, we show that unglycosylated PrP (ugPrP) is a *bona fide* ERAD substrate in yeast. Importantly, we demonstrate that ugPrP is a substrate of the Hrd3–Hrd1 proteolytic pathway in yeast. Increased levels of aberrant proteins lead to the Ire1-dependent upregulation of ER chaperones by the unfolded protein response (UPR), which promotes the refolding of immature ER proteins [22,23]. Although failure to degrade ugPrP does not elicit toxicity in *hrd1Δ* yeast cells, the expression of ugPrP or PrP leads to slow growth in *ire1Δ hrd1Δ* cells. These findings highlight the functions of ERAD and the UPR in regulating PrP activity and lay the foundation for defining the role of protein degradation in prion biology.

Material and methods

Yeast strains and plasmids. Cultures were grown in rich (YPD) or synthetic media containing standard ingredients and 2% glucose (SD medium), or 2% raffinose (SR medium), or 2% galactose (SG medium), or 2% raffinose + 2% galactose (SRG medium). Wild-type and yeast strains lacking *CUE1*, *DER1*, *ERV29*, *HRD1*, *DOA10*, *HRD3, UFD2*, and Hrd1-TAP strain were obtained from Open Biosystems (Huntsville, AL, USA). Strains MS193 (*Kar2-133*) and RSY801 (wild-type) were obtained from Dr. Jeff Brodsky. *Ire1Δ* and *hrd1Δ ire1Δ* mutants were provided by Dr. Thomas Sommer.

The plasmids containing human PrP wild-type and $PrP^{\Delta GPI}$ were constructed as previously described [18]. Human PrP was amplified by PCR and cloned to the 3′-end of the *GAL1* or *CPY* promoter in pRS416 for its expression. Mutations in the glycosylation sites (N181 and N197) were obtained using the Quick Change mutagenesis kit (Stratagene, Carlsbad, USA). The plasmids expressing human PrP and Ha-tagged Ub were obtained from Drs. Andrea LeBlanc and Mary Ann Osley, respectively.

Expression shut-off assay. Yeast cells, carrying plasmids that expressed PrP or its derivatives from the P_{GAL1} promoter, were grown at 30 °C to OD_{600} of ~0.5 in SR-ura medium with auxotrophic supplements and 3% raffinose as the carbon source. Expression of PrP or its derivatives was induced with galactose for 2 h and then repressed by the addition of 2% glucose. Samples were withdrawn at the indicated time points and harvested by centrifugation. Cells were resuspended in RIPA lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40,

0.5% sodium deoxycholate, 1% SDS, and protease inhibitor mix) and lysed by glass beads. Proteins were resolved by SDS–12% PAGE gel electrophoresis. Immunoblots were probed with monoclonal anti-PrP antibody 3F4 (Chemicon, Temecula, CA), then the goat anti-mouse HRP conjugate, and developed using ECL reagents (Amersham, Arlington Heights, IL). Treatment of yeast cells with 75 μM MG132 (Biomol, Plymouth meeting, PA) will be described elsewhere. The stable protein actin was used as a loading control to ensure that equal amounts of extracts were used in the experiments.

Detection of ubiquitylated ugPrP. Yeast cells expressing ugPrP and Ha-tagged Ub were grown in the galactose-containing SG medium to OD_{600} of ~1. Cells were lysed with glass beads in RIPA buffer. Lysates were mixed with beads linked to anti-PrP antibody 3F4 for 2 h at 4 °C. The immunoprecipitates were resolved by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with anti-Ha antibody (Covance, Berkeley, USA).

Cross-linking assay. Cells with or without TAP-tagged Hrd1, carrying either pRS416Gal-ugPrP or p416Gal vector, were grown in the SG-ura medium to mid-log phase. Cells were harvested by centrifugation and suspended in Mops buffer containing 0.4 mg/ml dithiobis(succinimidyl-propionate) (DSP) [24]. Cells were incubated for 30 min at 30 °C. Cells were then lysed and quenched with 20 mM hydroxylamine. Extracts were incubated with IgG Sepharose Fast Flow resin (Amersham Biosciences) for O/N and washed extensively with lysis buffer. Hrd1-containing complex was released from the beads with TEV protease (Invitrogen, Carlsbad, CA) at room temperature for 4 h. To further enrich Hrd1-binding proteins, elutes from the TEV reactions were then mixed with calmodulin beads (Stratagene) for 1 h at 4 °C. The immunoprecipitates were washed, treated with SDS to release bound proteins, which were resolved on SDS–PAGE, transferred, and then probed with anti-PrP antibody.

Results and discussion

Prion protein PrP and its derivatives are degraded in yeast

Several groups have successfully demonstrated that mammalian PrP can be efficiently targeted to the yeast ER after replacing its original ER-targeting signal sequence with an appropriate yeast ER signal sequence [16–18]. Following the approach utilized in one such study, we fused human PrP to a signal sequence derived from the yeast enzyme dipeptidyl aminopeptidase B (DPAPB) [18]. Consistent with mammalian studies, the DPAPB-PrP fusion is translocated to the ER, undergoes glycosylation, the attachment of glycophosphatidylinositol (GPI) moiety, and is then transported to the plasma membrane. To monitor PrP stability in yeast, the expression of DPAPB-PrP and its derivatives is controlled by a regulatable *GAL1* promoter, which can be induced by galactose and repressed by glucose. Consistent with earlier studies, PrP is expressed in yeast and can be glycosylated at two sites. Interestingly, after the shut-off of its transcription by the addition of glucose, all three forms (di-, mono-, and non-glycosylated) of PrP proteins are degraded in yeast (Fig. 1B).

To evaluate the role of the proteasome in PrP degradation, we examined the stability of PrP in the presence of the proteasome inhibitor MG132. The non-glycosylated PrP (g0) is significantly stabilized by MG132 (Figs. 1B and 2A), suggesting that g0 PrP is a substrate of the proteasome. Glycosylated PrP proteins are partially stabilized in MG132-treated cells. We found that glycosylated PrP proteins are completely stabilized in yeast when both the proteasome and lysosome are compromised (data not shown), suggesting that glyco-PrP proteins are degraded by the proteasome and lysosome, which is consistent with mammalian studies [5]. Our results also suggest that the degradation of PrP is likely conserved from yeast to human.

Next, we explored whether PrP degradation is dependent on post-translational modifications. In the ER, the GPI anchoring sequence (residues 232–254) is removed from PrP for the attachment of the GPI moiety (Fig. 1A), which anchors PrP to the plasma membrane. We found that deletion of the GPI sequence does not significantly alter the degradation of PrP in yeast (Fig. 1C), indicating that the membrane association is not critical for PrP turnover. Also in the ER, a subset of PrP is linked with N-linked sugar chains at one or two of its glycosylation sites N181 and N197. Glycosylation influences the degradation of some ERAD substrates (e.g., misfolded CPY) [25,26]. Proteins bearing a single mutation in either glycosylation site are degraded in yeast (Fig. 1D). The degradation of mono-glycosylated PrP is significantly reduced by the mutation in N197. Furthermore, unglycosylated PrP is also degraded in a proteasome-dependent manner (see below).

Unglycosylated PrP is degraded by ERAD in yeast

Since our results suggested that the degradation of the g0 form of PrP is proteasome-dependent (Fig. 1B), we decided to initially focus on the degradation of unglycosylated PrP (ugPrP) for the following reasons: (1) studying the degradation of all three forms of PrP is difficult because of the ongoing glycosylation and de-glycosylation *in vivo*; (2) although both glycosylated and unglycosylated PrPs are present in protein aggregates, unglycosylated PrP, by either *in vitro* synthesis or mutation of its glycosylation sites [27,28], can efficiently induce *in vivo* aggregate formation, suggesting that the unglycosylated form is critical for prion formation. Furthermore, the unglycosylated, but not glycosylated, PrP-derived peptide was found to easily form amyloid fibrils [29]; (3) in mammalian cells, MG132-induced accumulation of PrP is mostly non-glycosylated [11,13], although it could be due to deglycosylation in the cytosol; (4) a stop codon mutation in PrP, Y145stop, which should be devoid of glycosylation at residues 181 and 197, is rapidly degraded by the proteasome and associated with Gerstmann–Straussler–Scheinker syndrome, a familiar TSE [30].

To study ugPrP degradation, we mutated two glycosylation sites in PrP to create ugPrP. ugPrP is degraded in wild-type cells but stabilized in the presence of MG132 (Fig. 2A), indicating that ugPrP is a substrate of the proteasome. Is ugPrP subjected to ERAD? We assayed the stability of ugPrP in various mutants defective in ERAD [9,10]. Two ER membrane proteins, Cue1 (anchoring E2 enzymes to the ER membrane) and Der1 (ER-cytosol

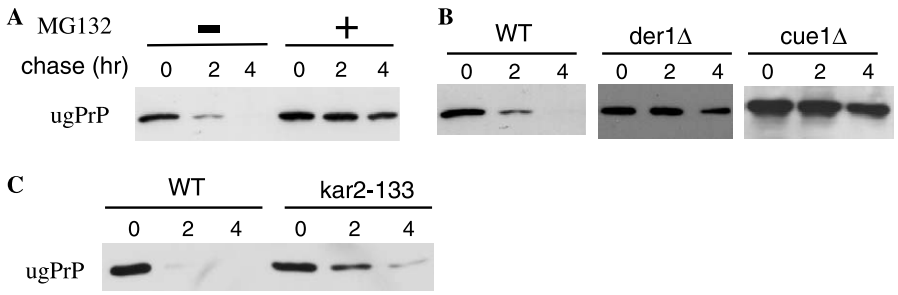

Fig. 2. Unglycosylated PrP is degraded by ERAD in yeast. (A) Unglycosylated PrP (ugPrP) is degraded by the proteasome. Two glycosylation sites in PrP were simultaneously mutated, N181Q and N197Q, to abolish the glycosylation. Wild-type cells containing a *GAL1* promoter regulated ugPrP were first grown in raffinose containing media. Cells were treated with or without 75 μM MG132. Expression of ugPrP was induced by the addition of galactose. Samples were taken after promoter shut-off at intervals and analyzed by anti-PrP Western blots. (B) Degradation of ugPrP is impaired in *der1* and *cue1* mutants. Expression shut-off assay in isogenic wild-type, *der1Δ*, *cue1Δ* strains was conducted as described in (A). (C) ugPrP degradation is compromised in kar2 mutant. ugPrP stability was determined by expression shut-off assay in wild-type and *kar2-133* temperature sensitive mutant. To inactivate Kar2 function, cells were shifted to 37 °C for 30 min before the addition of galactose and kept at 37 °C.

translocation), are required for the degradation of many ERAD substrates. We found that ugPrP degradation was markedly compromised in *der1* and *cue1* mutants (Fig. 2B), indicating that ugPrP is an ERAD substrate in yeast. These results suggest that unglycosylated PrP is recognized as a misfolded protein in the ER and degraded by ERAD. Previous studies in mammalian cells indicate that the g0 form of PrP is stabilized upon the treatment of proteasome inhibitor [6–8]. However, the identity of the g0 form of PrP was not clear. Sugar chains are removed from some glycosylated ERAD substrates in the cytosol prior to proteasome-mediated degradation [31]. The g0 form of PrP stabilized could be a mixture of both un-glycosylated and de-glycosylated PrP. Our results directly demonstrate that unglycosylated PrP is a ERAD substrate.

ER chaperones, such as BiP, promote the degradation of a subset of soluble ERAD substrates [10]. We investigated whether BiP (called Kar2 in yeast) is involved in ugPrP turnover. The plasmid bearing ugPrP was transformed into cells containing the *kar2-133* allele, in which Kar2 function can be compromised by shifting the growth temperature to 37 °C. We found that ugPrP is degraded faster in wild-type cells at 37 °C, while ugPrP degradation is impaired in cells with reduced Kar2 activity (Fig. 2C), suggesting a role for BiP in ugPrP turnover.

Unglycosylated PrP is a substrate of the Hrd1–Hrd3 pathway

Three ERAD pathways exist in yeast (Fig. 3A). To determine the specific route for PrP degradation, we evaluated ugPrP degradation in yeast cells defective in each degradation pathway. ugPrP degradation was unaltered in cells lacking *ERV29* (ER to Golgi transport) (Fig. 3B), which encodes a central component of the HIP pathway (Hrd-independent pathway) [32]. Doa10 and Hrd1 are two E3 enzymes required for the degradation of the majority of ERAD substrates [10]. While Doa10 is a single component E3, Hrd3, and Hrd1 (a RING finger containing protein) form an E3 complex. ugPrP is stabilized in *hrd3Δ* (Fig. 3C), but not *doa10Δ* (Fig. 3C), suggesting that ugPrP is a substrate of the Hrd1–Hrd3 pathway. Some Hrd3-regulated ERAD substrates (e.g., Hmg2) require Ufd2, a Ub chain elongation factor (E4). We found that ugPrP degradation is Ufd2 dependent (Fig. 3C). Interestingly, Ufd2A, one of the Ufd2 mouse homologs, is highly expressed in neuronal cells and protects neurons from ER stress-induced degeneration [33].

The E3 component plays a critical role in targeting substrates for destruction. E3 enzymes directly bind substrates and catalyze the Ub chain assembly onto substrates. To ascertain the role of Hrd1 and Hrd3 in ugPrP degradation, we compared the ubiquitylation pattern of ugPrP in wild-type, *doa10Δ*, *hrd3Δ*, and *hrd1Δ* cells. To this end, we co-transformed the plasmid expressing ugPrP and the plasmid bearing Ha-tagged Ub to these cells. Ubiquitylated ugPrP species were seen in wild-type cells expressing both ugPrP and Ha-Ub, but not in control cells lacking either ugPrP or Ha-Ub (Fig. 3D). Ubiquitylated ugPrP bands were absent in *hrd1Δ* and *hrd3Δ* cells, but present in the *doa10Δ* mutant (Fig. 3D).

Does ugPrP bind the Hrd1–Hrd3 E3 complex? To this end, we employed a cross-linking strategy, which was used successfully to detect the bindings of several ERAD substrates to E3s involved in ERAD (e.g., Hrd1, Doa10) [24]. Specifically, we introduced the plasmid bearing ugPrP into the Hrd1-TAP strain [34], in which the chromosomal copy of *HRD1* is linked to the TAP (tandem affinity purification) tag that consists of calmodulin binding peptide (CBP), a TEV protease cleavage site, and IgG binding domain. Extracts were suspended in Mops buffer with dithiobis(succinimidyl-propionate), a cross-linking reagent. Hrd1-binding proteins were enriched by immunoprecipitations. The immunoprecipitates were transferred to membrane and then probed with anti-PrP antibody. We found that ugPrP binds Hrd1-TAP specifically (Fig. 3E). These

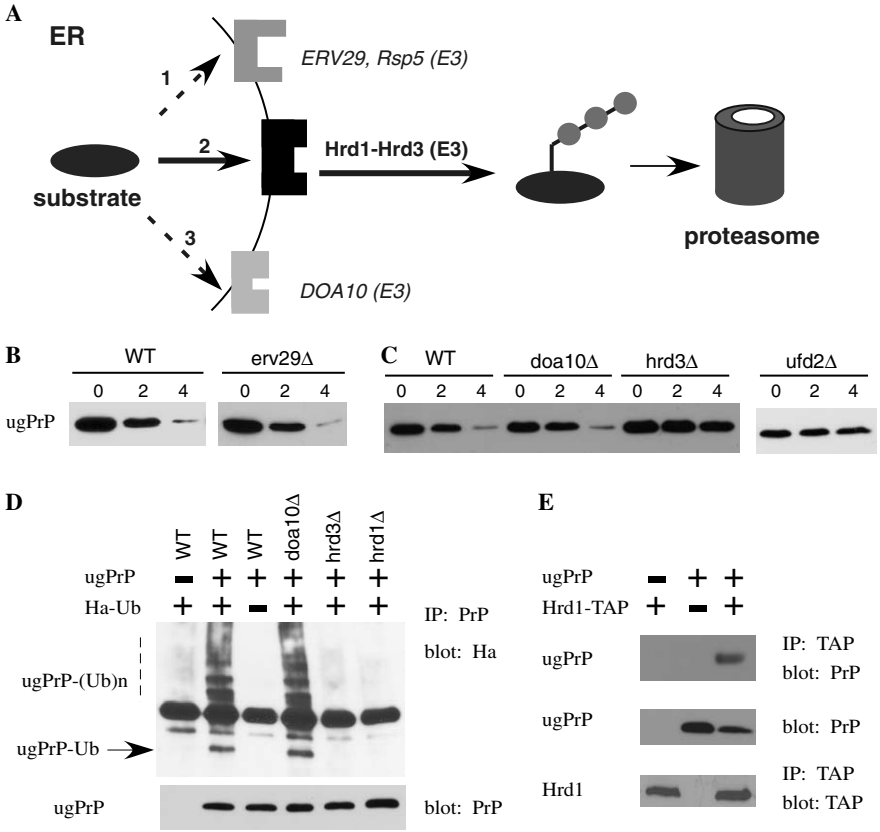

Fig. 3. ugPrP is degraded by the Hrd1–Hrd3 pathway. (A) Multiple ERAD pathways in yeast. Doa10 and the Hrd1–Hrd3 complex are E3s bound to the ER membrane [10]. Some ERAD substrates are degraded by a pathway dependent on Erv29 (an ER membrane protein), and Rsp5 (E3) [32]. (B,C) Degradation of ugPrP in the indicated strains. ugPrP stability was determined as in (Fig. 2A). (D) Hrd1 and Hrd3 are required for ugPrP ubiquitylation. ugPrP was precipitated with anti-PrP antibody and analyzed by immunoblotting with anti-Ha antibody. Ubiquitylated bands are indicated on the left of the upper panel. The bottom panel shows the levels of ugPrP in these cells. The antibodies used for immunoprecipitation (IP) and Western blot (blot) are indicated on the right of the panels. (E) Hrd1 binds ugPrP. Cells expressing indicated proteins were treated with the cross-linker DSP. Proteins extracted from these cells were incubated with IgG Sepharose. Bound proteins were released by the TEV protease, and subsequently mixed with calmodulin Sepharose to isolate proteins associated with Hrd1-TAP. Hrd1-binding proteins were separated on SDS–PAGE and immunoblotted for cross-linked ugPrP with anti-PrP antibody.

combined results indicate that ugPrP is a substrate of the Hrd1–Hrd3 E3 complex in yeast.

Growth defects in ire1Δ hrd1Δ cells expressing ugPrP or PrP

How important is ugPrP turnover? PrP-induced toxicity has not been previously observed in yeast, which could be due to continuous degradation of PrP. Hence, we compared the growth of wild-type and *hrd1Δ* cells overexpressing ugPrP or PrP upon galactose induction. We found that the accumulation of ugPrP in *hrd1Δ* cells did not significantly affect cell growth (Fig. 4A and B).

The accumulation of misfolded secretory proteins triggers a signaling pathway—the unfolded protein response (UPR), which includes transcriptional induction of ER chaperones [23,35]. The resulting UPR activation promotes the refolding of misfolded proteins. A key regulator of the UPR is the ER stress sensor protein Ire1, which has both protein kinase activity and endonuclease activity [22]. Ire1 activates a transcription factor Hac1 and subsequent expression of Hac1-regulated UPR target genes. To determine the importance of the UPR in regulating PrP, we transformed the plasmid expressing ugPrP, or PrP to *ire1Δ*, and *ire1Δ hrd1Δ* mutants. Interestingly, expression of ugPrP or PrP led to slower growth (~4-fold) in UPR-deficient *ire1Δ* mutant (Fig. 4C). More severe growth impairment (>16-fold) was caused by additional mutation in Hrd1 (Fig. 4D). It is interesting that PrP and ugPrP have similar effects on cell growth (Fig. 4D). The data suggest that UPR and ERAD are involved in cellular tolerance of PrP and ugPrP.

A concern of our results is that the *GAL1* promoter regulated PrP expression may be too high, which may lead to artifacts. We made the construct expressing ugPrP or PrP under the regulation of a weaker, constitutive promoter derived from yeast carboxypeptidase (CPY). Misfolded CPY is a classic ERAD substrate that has been used extensively [10]. Expression of this P_{CPY}-ugPrP allele is very low in wild-type cells, but significantly higher in *hrd1Δ* mutant (Fig. 4E). The results support the requirement of Hrd1 for ugPrP degradation (Fig. 3). We also introduced P_{CPY}-ugPrP and P_{CPY}-PrP alleles to various yeast mutants

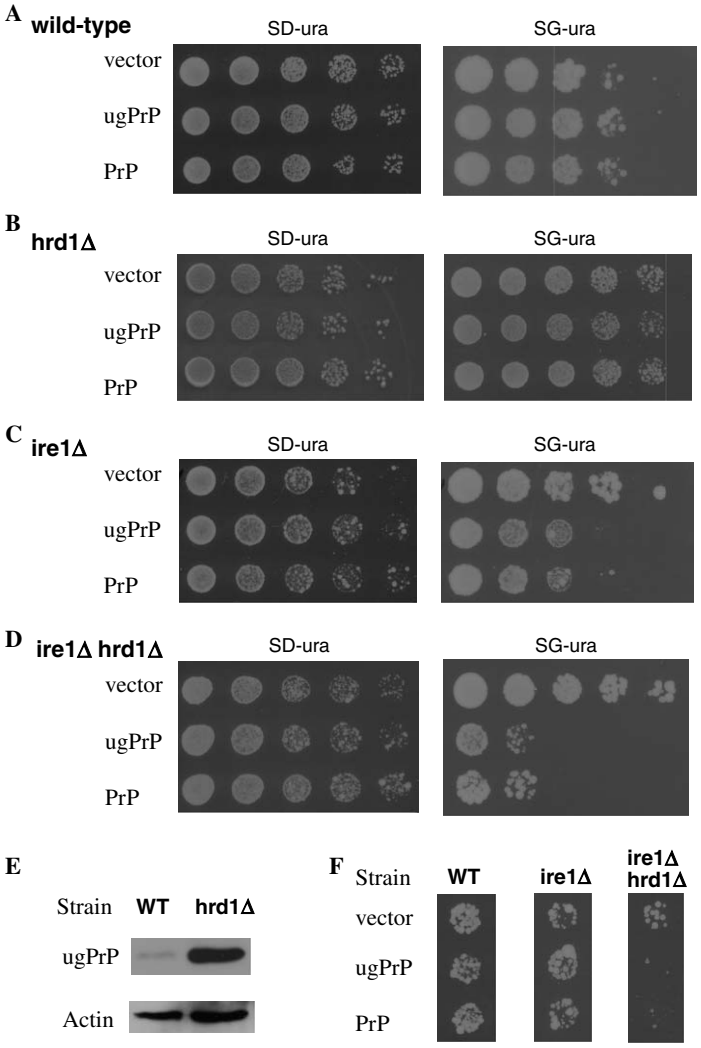

Fig. 4. The UPR and ERAD deficient *ire1Δ hrd1Δ* cells are sensitive to the accumulation of ugPrP or PrP. (A–D) PrP, ugPrP and a control vector were separately introduced to wild-type, *hrd1Δ*, *ire1Δ*, and *hrd1Δ ire1Δ* cells as indicated. Exponentially growing yeast cells were spotted onto glucose-containing SD-ura (expression off) and galactose-containing SG-ura (expression on) media in serial 4-fold dilutions. The plates were incubated at 30 °C for 2–5 days. The transformed plasmids are labeled on the left of the panel. (E) Accumulation of constitutive CPY promoter-regulated ugPrP in *hrd1Δ* cells. The steady-state level of ugPrP was determined by Western blot analysis in wild-type and *hrd1Δ* cells. Protein extracts were separated on SDS–PAGE, transferred to PVDF membrane, and then probed with the anti-PrP antibody to detect ugPrP. As a loading control, we probed the blot with anti-actin antibody. The identity of the bands is indicated on the left. The strains are indicated at the top of the panel. (F) Expression of the CPY promoter regulated PrP or ugPrP in yeast. A vector, the CPY promoter regulated PrP and ugPrP were separately transformed to wild-type, *ire1Δ*, and *hrd1Δ ire1Δ* cells as indicated, and spotted onto SD-ura plates in serial dilutions. The representative spots are shown. PrP or ugPrP expression caused no slow growth in wild-type, *ire1Δ* cells, ~4-fold slower growth in *hrd1Δ ire1Δ* cells.

(Fig. 4F). Consistent with decreased expression level using the CPY promoter, the PrP-induced phenotypes in yeast were reduced. Normal growth was seen in PrP expressing *ire1Δ* or *hrd1Δ* cells (Fig. 4F and data not shown). The PrP-induced slower growth (~4-fold) in *ire1Δ hrd1Δ* cells still exists (Fig. 4F). It is impossible to compare the levels of P_{CPY}-PrP in yeast and endogenous PrP in aging brains. Our results suggest that both UPR and ERAD are involved in PrP regulation.

How and why PrP may cause neurodegeneration remains elusive. Some of the perplexing issues are: Why are little PrP^{Sc} aggregates found in some prion diseases [36–39]? Why are the incubation time and lesions different in various prion-related disorders [4,39]? Why does PrP^{Sc} induce death in the brain but not other organs [4,38,39]? Multiple mechanisms, including PrP^{Sc}-independent, may account for various prion-induced cell death. It is possible that compromised UPR activity and proteolysis could be two, but not the only, contributing factors in some prion diseases.

In mammals, accumulation of PrP via proteasome inhibition has been proposed to directly lead to cytotoxicity [11]. However, this model has recently been challenged by conflicting results from several laboratories [12–15]. Furthermore, besides PrP, proteasome inhibition likely blocks the degradation of many proteins, some of which may be

involved in neurodegeneration and contribute to cell death. Therefore, the mechanism underlying PrP-induced toxicity remains unclear. Ire1-regulated UPR signaling pathway is conserved in mammals [22]. Our findings suggest that reduced UPR activity may play a role in the pathophysiology of some prion-diseases. ER stress-triggered apoptosis is proposed as a crucial mechanism for neuronal cell death in neurodegenerative disorders [22,23]. Relevant to this notion is that proteasome activity declines with age and is compromised in many neurodegenerative disorders [2]. It will be important to determine whether the activities of UPR and ERAD are down-regulated in old cells and prion patients.

Our results suggest that yeast is an excellent model organism to dissect the degradation mechanism of PrP. In this paper, we show that ugPrP is an ERAD substrate in yeast. We find that ugPrP degradation is mediated by a pathway consisting of highly conserved proteins including an ER chaperone Kar2, a transmembrane protein Der1 that may be a channel for protein translocation, and a Ub-protein ligase (E3) complex of Hrd1 and Hrd3. Homologs of these proteins may regulate PrP degradation in higher eukaryotes, which is being pursued in our laboratory. It is noteworthy that Hrd1 and its ortholog gp78 are required for the degradation of yeast and human Hmg-CoA reductase [40], respectively.

The data provide new insights regarding the mechanism governing PrP degradation and lay the foundation for uncovering the roles of ERAD and UPR in prion biogenesis and pathology. Various genetic and biochemical means would be used to uncover additional genes critical for the degradation of unglycosylated and glycosylated PrP. Using yeast genetics, suppressors of PrP-induced growth defects in an *ire1Δ hrd1Δ* background could be isolated and may reveal effective approaches to prion-related diseases.

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