

## RESEARCH ARTICLE

# 4-Phenylbutyrate suppresses the unfolded protein response without restoring protein folding in *Saccharomyces cerevisiae*

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**One sentence summary:** The chemical chaperone 4-phenylbutyrate suppresses the unfolded protein response without restoring the protein-folding status in the endoplasmic reticulum.

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## ABSTRACT

Accumulation of unfolded secretory proteins in the endoplasmic reticulum (ER), namely ER stress, is hazardous to eukaryotic cells and promotes the unfolded protein response (UPR). Ire1 is an ER-located transmembrane protein that senses ER stress and triggers the UPR. According to previous *in vitro* experiments, 4-phenylbutyrate (4-PBA) works as a chemical molecular chaperone. Since 4-PBA attenuates the UPR in mammalian tissue cultures, this chemical may have clinical potential for restoring ER-stressing conditions. In this study, we investigated 4-PBA's mode of action using the yeast *Saccharomyces cerevisiae* as a model organism. Although 4-PBA blocked a dithiothreitol (DTT)-induced UPR, it did not appear to restore impairment of ER protein folding that was caused by DTT. Moreover, even under non-stress conditions, 4-PBA attenuated UPR that was induced by an Ire1 mutant that exhibits a substantial activity without sensing ER accumulation of unfolded proteins. We also found that 4-PBA drastically promotes the degradation of Ire1. These observations indicate that at least in the case of yeast cells, 4-PBA suppresses the UPR not through restoration of the ER function to correctly fold proteins. Instead, the accelerated degradation of Ire1 possibly explains the reason why the UPR is attenuated by 4-PBA.

**Keywords:** 4-phenylbutyrate; molecular chaperone; unfolded protein response; endoplasmic reticulum; protein folding; drug side effects

## INTRODUCTION

The endoplasmic reticulum (ER) is the cellular compartment where secretory and transmembrane proteins are folded. Misfolding of proteins can be hazardous to cells through various reasons. Aberrant ER client proteins, such as mutants of cystic fibrosis transmembrane conductance regulator (CFTR), are

unable to reach their final destination and exert their proper function because they are accumulated in the ER and degraded through the ER-associated protein degradation (Pranke and Sermet-Gaudelus 2014). Moreover, misfolded proteins and their aggregates per se are toxic to cells (Soto 2003). The ER stress response or the unfolded protein response (UPR) is triggered by

ER accumulation of misfolded proteins, so-called ER stress, commonly in eukaryotic organisms (Mori 2009; Walter and Ron 2011). Although the ER stress response generally protects cells against protein aggregation, in the case of metazoan cells, it can also lead to apoptosis (Shore, Papa and Oakes 2011). Because these issues are tightly related to various human diseases, which include diabetic mellitus and some neurodegenerative diseases, chemical compounds that suppress ER protein aggregation and the ER stress response may be valuable sources for drug development (Oakes and Papa 2015).

It is well known that 4-phenylbutyrate (4-PBA) is such a compound (Kolb et al. 2015; Ranga Rao et al. 2018). While being soluble in aqueous environments, 4-PBA has a hydrophobic moiety, implying that it can inhibit aggregation of proteins as a chemical chaperone. Intriguingly, 4-PBA suppresses the UPR in both mammalian and yeast cells (Kubota et al. 2006; Ozcan et al. 2006; Pineau et al. 2009; Le et al. 2016).

Ire1 is an ER-located type-I transmembrane protein commonly found in all eukaryotic species. In the budding yeast *Saccharomyces cerevisiae*, Ire1 is believed to serve as a sole ER-stress sensor that initiates the UPR, which is a dramatic transcriptome change that occurs in response to ER stress. Ire1 has an endoribonuclease domain in its cytosolic moiety (Sidrauski and Walter 1997). When yeast cells are exposed to ER stress, Ire1 promotes the splicing of the *HAC1* mRNA (Cox and Walter 1996), which is then translated into a transcription factor protein that changes the yeast transcriptome to restore ER functions (Travers et al. 2000; Kimata et al. 2006).

As reviewed in our previous publication (Kimata and Kohno 2011), the luminal domain of Ire1 plays critical roles in regulating the activity of Ire1 in response to ER stress. The ER-located molecular chaperone BiP is associated with the luminal domain of Ire1 to inhibit self-association and activation of Ire1 (Bertolotti et al. 2000; Okamura et al. 2000; Kimata et al. 2003). ER-accumulated misfolded proteins cause dissociation of BiP from Ire1 and enable the self-association of Ire1, which alone is not sufficient for its activation (Kimata et al. 2004; Oikawa, Kimata and Kohno 2007). We and others have also reported that unfolded proteins are directly captured by the luminal domain of Ire1 for complete induction of the UPR (Kimata et al. 2007; Gardner and Walter 2011; Promlek et al. 2011).

These insights explain the molecular mechanism by which ER-accumulated misfolded proteins activate Ire1 and trigger the UPR. It is thus widely believed that 4-PBA may attenuate the UPR by restoring the protein-folding status in the ER. Indeed, 4-PBA is likely to assist the proper folding of ER client proteins such as a CFTR mutant (Rubenstein, Egan and Zeitlin 1997; Lim et al. 2004; Kubota et al. 2006).

In this study, using yeast as a model organism, we addressed the molecular process through which 4-PBA suppresses the UPR. Our findings indicate that contrary to the theory described above, 4-PBA does not seem to assist protein folding in the ER. Here we propose that 4-PBA induces the degradation of Ire1, which is likely to result in the attenuation of the UPR.

## MATERIAL AND METHODS

### Yeast strains

*Saccharomyces cerevisiae* *ire1Δ* (*ire1::TRP1*) strain KMY1015 was derived from a standard laboratory strain KMY1005 (*MATα leu2-3112 ura3-52 his3Δ200 trp1Δ901 lys2-801*) and transformed with the UPR lacZ-reporter plasmid pCZY1 (Mori et al. 1992). The resulting strain was further transformed with a single copy plas-

mid pRS313-IRE1 (Kimata et al. 2004), which has the endogenous IRE1 gene (the coding sequence plus the 5'- and 3'-untranslated regions), and was used as a wild-type IRE1 strain TKY001. Strain TKY002 is a derivative of TKY001 that has the bZIP mutant version of the IRE1 gene (Promlek et al. 2011) instead of the wild-type IRE1 gene. Strain TKY003 was obtained by transforming KMY1005 with the *eroGFP* expression plasmid pPM28 (Merkamer, Trusina and Papa 2008).

Insertion of an HA epitope sequence into the IRE1 gene was previously described (Kimata et al. 2003). The resulting gene was inserted into the single copy plasmid vector (to generate pRS315-IRE1-HA; Kimata et al. 2004) or into the multicopy plasmid vector (to generate pRS423-IRE1-HA; Kimata et al. 2003) and was used for expression of the C-terminally HA-tagged version of Ire1 (Ire1-HA). We transformed KMY1015 with pCZY1 and pRS315-IRE1-HA to obtain strain TKY004 and with pCZY1 and pRS423-IRE1-HA to obtain strain TKY005. TKY006 is a derivative of TKY004 that has the bZIP mutant version of the IRE1-HA gene (Promlek et al. 2011).

Strain BY4742*hrd1Δ* (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hrd1::kanMX4*) was purchased from EUROSCARF (<http://www.euroscarf.de>). The *DOA10* gene on BY4742*hrd1Δ* was disrupted by the *HIS5MX* marker, and the resulting *hrd1Δ doa10Δ* strain was further transformed with pRS315-IRE1-HA and named TKY007.

For all experiments shown in this study, yeast cells were cultured at 30°C in standard synthetic dextrose medium containing 2% glucose, 0.66% yeast nitrogen base without amino acids (MP Biomedicals) and appropriate auxotrophic requirements.

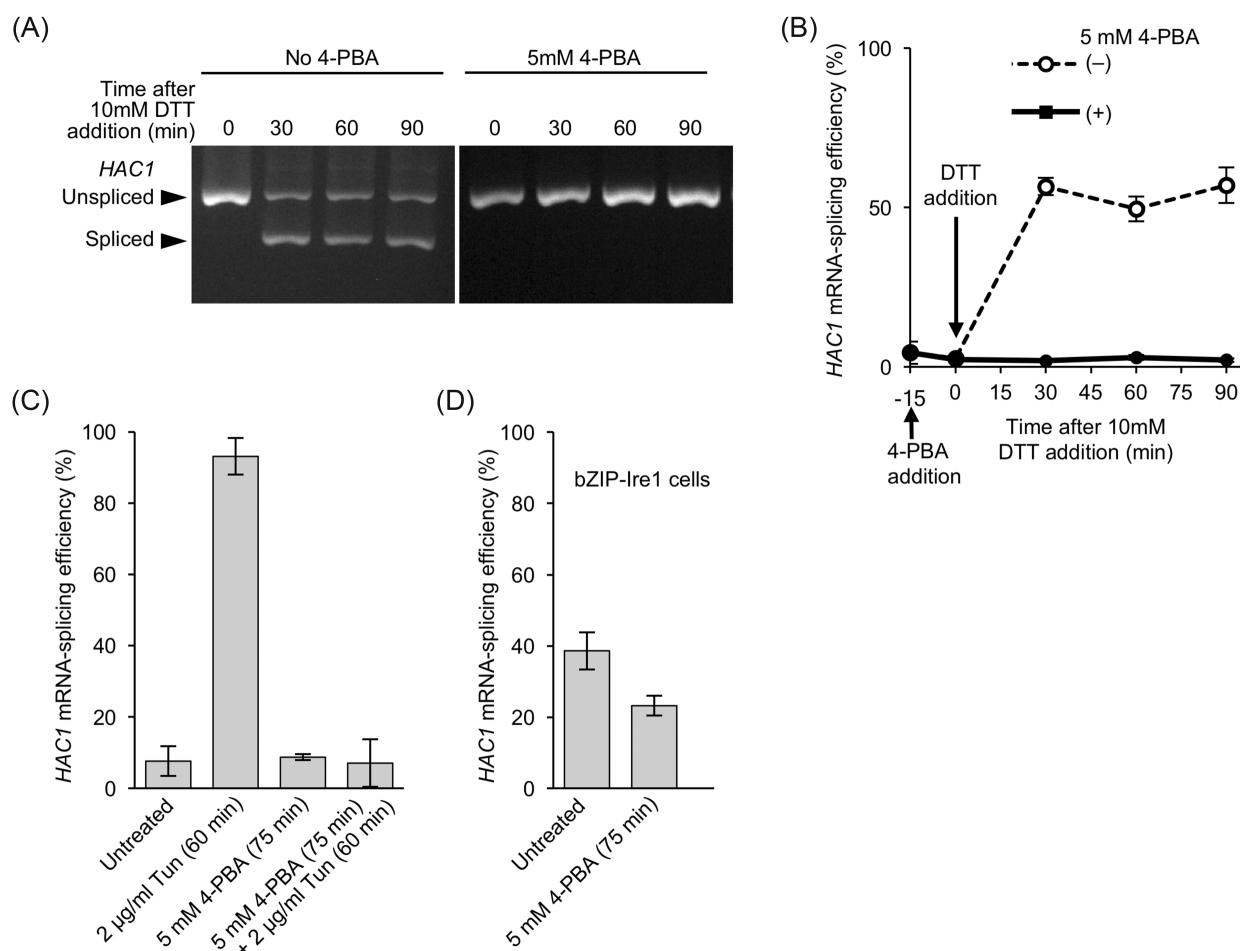
### Chemicals

Dithiothreitol (DTT; catalog code: D0632), tunicamycin (catalog code: T7765), and 4-PBA (catalog code: SML0309) were purchased from Sigma-Aldrich.

### Protein analyses

After being harvested by centrifugation at 1600 × g for 1 min, 5.0 OD<sub>600</sub> cells were disrupted by agitation with glass beads (425–600 μm) in 100 μl of the lysis buffer containing 50 mM Tris-Cl (pH 7.9), 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100 and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 20 μg/ml pepstatin A and Calbiochem Protease Inhibitor cocktail Set III (100× dilution)). The cell lysates were then clarified by centrifugation at 8000 × g for 10 min, fractionated using standard SDS-DTT polyacrylamide gel electrophoresis (PAGE), and transferred onto polyvinylidene difluoride-based western blotting membrane (Hybond-P; GE Healthcare). The semi-dry electrophoretic transfer and subsequent treatment of the blot membranes were performed as indicated by the manufacturer's instruction. As primary antibodies, we used 12CA5 mouse monoclonal anti-HA antibody (Roche), 22C5D8 mouse monoclonal anti-Pgk1 antibody (Abcam) and rabbit polyclonal anti-yeast BiP antibody (Kimata et al. 2003). After treatment of the blot membranes with the horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) reagent (ECL prime, GE healthcare), the ECL signal was quantitatively detected using the luminescence imager LAS4000 (Fuji Film).

For the BiP aggregation assay, cells were disrupted as described in the previous paragraph and subjected to sequential centrifugations at 720 × g for 30 s and 8000 × g for 15 min. The



**Figure 1.** Attenuation of the *HAC1* mRNA splicing by 4-PBA. (A) We added (or did not add) 4-PBA in the cultures of the wild-type *IRE1* strain TKY001, and after 15 min, DTT was added. The cells were then harvested at the indicated time points, and their total RNA samples were analyzed through RT-PCR amplification of the *HAC1* transcripts, which was followed by agarose gel electrophoresis and ethidium bromide staining. (B) Data from the experiment shown in panel A are numerically expressed as the *HAC1* mRNA-splicing efficiency. (C) A similar experiment as in panels A and B was performed using tunicamycin instead of DTT. For dual treatment with 4-PBA and tunicamycin, tunicamycin was added into the cultures 15 min after adding 4-PBA. (D) After culturing with 4-PBA or under untreated conditions, TKY002 cells that had bZIP-Ire1 were assessed for *HAC1* mRNA splicing.

pellet fractions obtained by the second centrifugation were then washed twice using the lysis buffer and analyzed as the 'pellet' samples with the anti-BiP western blotting.

For the pulse-chase experiment, cells were labeled by culturing with  $^{35}\text{S}$ -labeled methionine and cysteine (400 kBq/ml; EXPRE $^{35}\text{S}$  Protein Labeling Mix, PerkinElmer) for 2 h. Chase incubation was initiated by adding non-labeled methionine at a final concentration of 0.004% and cysteine at a final concentration of 0.003%. Cell lysates were then obtained as mentioned above and subjected to anti-HA immunoprecipitation, as previously described (Kimata et al. 2003). Standard SDS-DTT PAGE was performed to fractionate the immunoprecipitation products, and the radioactive signal was detected on an autoradiograph. For autoradiography, a storage phosphor screen BAS-MS2040 (Fuji Film) and its reader BAS2500 (Fuji Film) were employed.

### RNA analyses

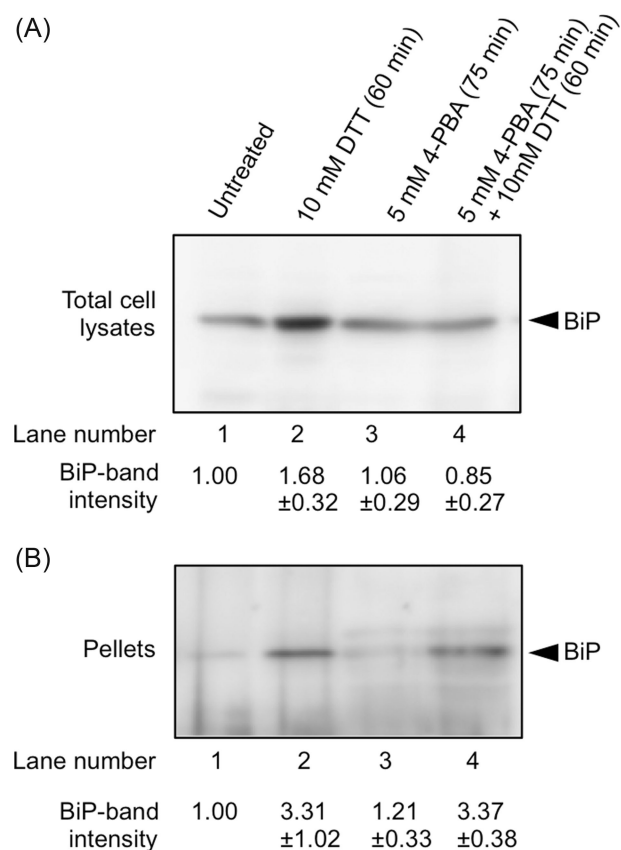
The hot phenol method was employed for extracting total RNA from yeast cells (Kimata et al. 2003). To monitor the *HAC1* mRNA splicing, we subjected the total RNA sample to reverse transcriptase (RT)-PCR analysis using a 17-mer polyA primer for the RT reaction and the *HAC1*-specific primer sets for PCR

(Promlek et al. 2011). The resulting RT-PCR products were then run on a 2% agarose gel, stained with ethidium bromide and observed with the fluorescence imager GelDoc XR + system (BioRad).

For quantification of the *IRE1*-gene transcript, total RNA samples were subjected to RT reaction with the 17-mer polyA primer. We then analyzed the RT reaction products by qPCR using SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara) in the real-time PCR machine LightCycler 96 (Roche). The primer sets used are as follows: 5'-AGT CAG AAT TTT CCA TCT TTA GTC G-3'/5'-CGG TCA CTG GAA GCG TAT CT-3' for the amplification of the *IRE1*-gene product and 5'-ATA TTC CAG GAT CAG GTC TTC CGT AGC-3'/5'-GTA GTC TTC TCA TTC TGT TGA TGT TGT TGT TG-3' for the amplification of the *TAF10*-gene product, which served as an internal control.

### Microscopic observation

We observed yeast cells carrying the *eroGFP* expression plasmid using the Keyence BZ-9000E microscope and the objective lens CFI Plan Apo  $\lambda$ 100xH (Nikon). Florescence images were observed using the filter set OP-79301 (excitation wavelength, 472.5/30; dichroic mirror wavelength, 495; emission, 520/35) for



**Figure 2.** 4-PBA does not diminish BiP sedimentation induced by cellular treatment with DTT. The IRE1 cells TKY001 were cultured with 4-PBA and/or DTT (or none of both (untreated)), as in Fig. 1A and B. For dual treatment with 4-PBA and DTT, DTT was added into the cultures 15 min after adding 4-PBA. Total cell lysates were then obtained and fractionated by centrifugation for the BiP sedimentation assay. Panels A and B represent anti-BiP western blot images of the total cell lysates (equivalent to 0.05 OD<sub>600</sub> cells) and the pellet samples (equivalent to 1.1 OD<sub>600</sub> cells), respectively. Band densities were then quantified and normalized against that of the untreated samples, which are set at 1.00, before calculation of the means and standard deviations of the values from multiple cultures.

excitation with blue light (1.0-s exposure) and a custom-made filter set (excitation wavelength, 395/25; dichroic mirror wavelength, 495; emission, 510/20) for excitation with UV/violet light (1.3-s exposure).

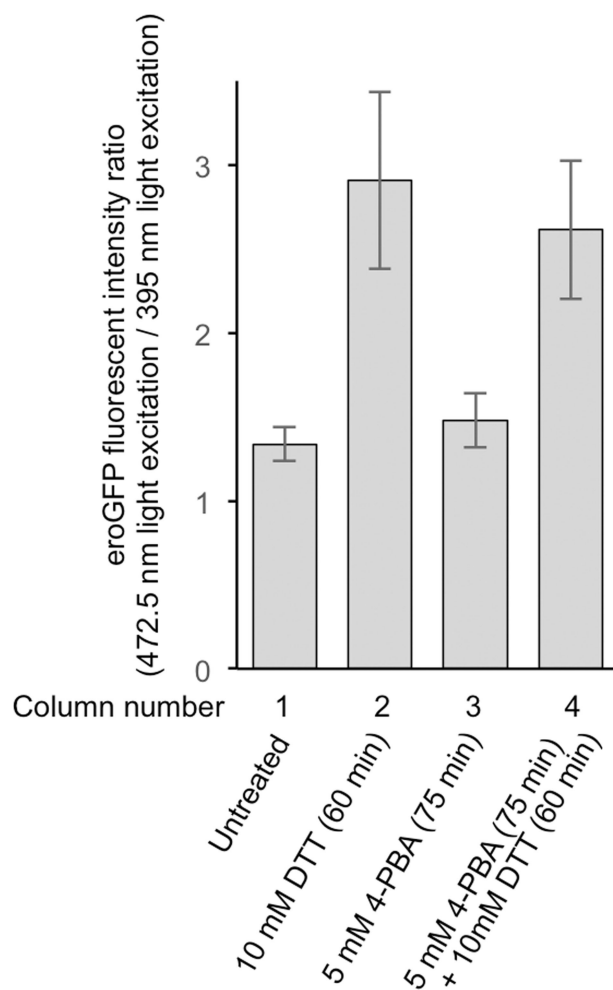
### Data quantification

In this study, we analyzed gel images using the image processing software Image J (<https://imagej.nih.gov/ij/>) to obtain quantitative data, which are presented here as averages and standard deviations of the values from multiple cultures. The following formula was used to obtain the 'HAC1 mRNA-splicing efficiency' from fluorescent images of the HAC1 RT-PCR products:  $\frac{[(\text{band intensity of the spliced form}) / \{(\text{band intensity of the spliced form}) + (\text{band intensity of the unspliced form})\}]}{}$ .

## RESULTS

### 4-PBA suppresses yeast UPR.

In this study, we monitored the UPR in yeast cells through checking the HAC1-mRNA splicing, which was measured using the RT-

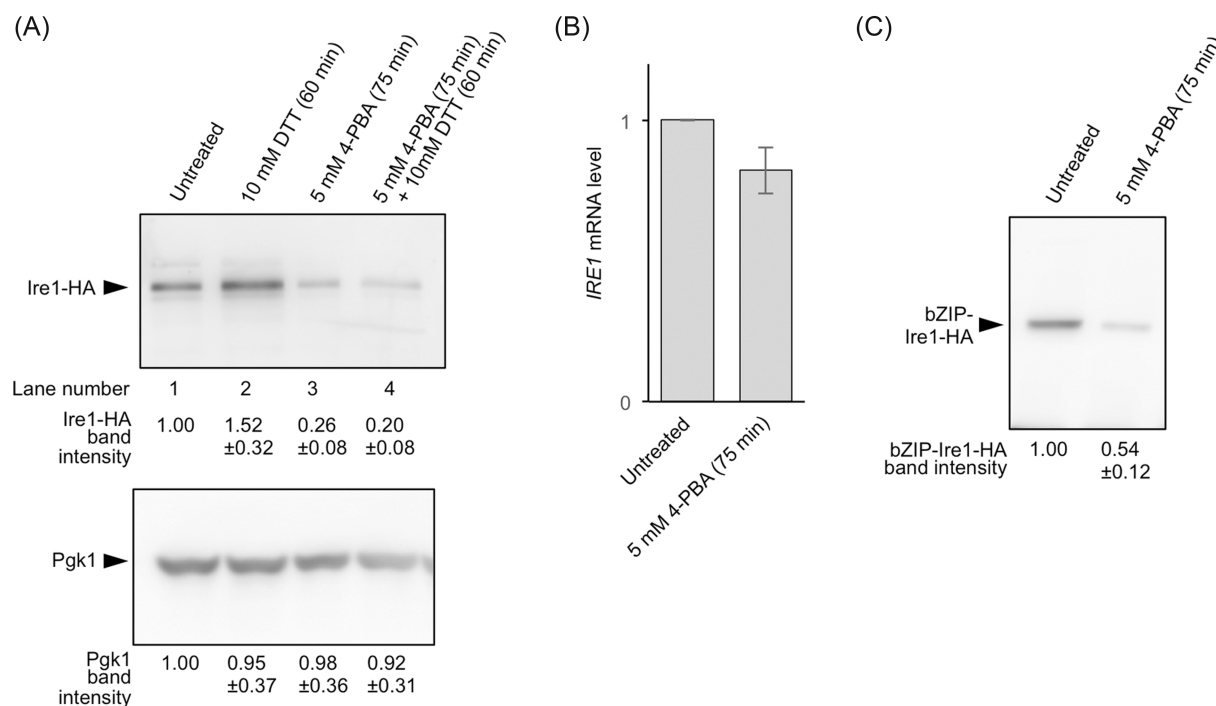


**Figure 3.** DTT impairs disulfide-bond formation in the ER even in the presence of 4-PBA. The IRE1 cells TKY003 producing eroGFP were cultured with 4-PBA and/or DTT (or none of both (untreated)), as in Fig. 1A and B, and then observed under a fluorescent microscope as described in the Materials and Method section. For dual treatment with 4-PBA and DTT, DTT was added into the cultures 15 min after adding 4-PBA. Fluorescent intensities of a cell illuminated by UV/violet light (395 nm) and blue light (472.5 nm) were quantified (100 cells were observed in each sample) and used for the calculation of the indicated values.

PCR technique that amplifies the HAC1 mRNA species. In the experiment shown in Fig. 1A and B, yeast cells were cultured in the presence of 10 mM DTT, a disulfide-reducing reagent that damages ER protein folding and causes a potent UPR. As we reported previously (Le et al. 2016), 5 mM 4-PBA almost completely abolished the UPR when added into the culture 15 min before DTT imposition. 4-PBA also attenuates the UPR induced by an N-glycosylation inhibitor tunicamycin (Fig. 1C), as initially indicated by Pineau et al. (2009).

In a mutant form of yeast Ire1, which is named as bZIP-Ire1, the full-length luminal domain was replaced with a basic leucine zipper (bZIP) dimer-forming peptide that was derived from the yeast bZIP-family transcription factor protein Gcn4 (Liu, Schroder and Kaufman 2000; Promlek et al. 2011). This mutant is located on the ER membrane and exhibits a modest UPR-inducing activity even in the absence of external ER-stressing stimuli. As shown in Fig. 1D, 4-PBA attenuated, although not drastically, the HAC1-mRNA splicing induced by bZIP-Ire1 in non-stressed yeast cells.





**Figure 4.** A decrease of Ire1 in 4-PBA-treated cells at the protein level but not at the mRNA level. (A) Yeast cells producing Ire1-HA (TKY004) were cultured with 4-PBA and/or DTT (or none of both (untreated)), as performed in Fig. 1A and B. For dual treatment with 4-PBA and DTT, DTT was added into the cultures 15 min after adding 4-PBA. Total cell lysates (equivalent to 0.13 OD<sub>600</sub> cells) were then analyzed through anti-HA and anti-Pgk1 western blotting. Band densities were quantified and are presented as in Fig. 2. (B) Wild-type IRE1 cells (TKY001) were cultured with 4-PBA or remained untreated for measuring the cellular amount of the IRE1-gene transcript. The data were obtained as relative values against the cellular TAF10-mRNA levels, which served as inner controls, and normalized against the value of the untreated sample, which is set at 1.00. (C) A similar assay as performed in panel A was performed using yeast cells producing the bZIP mutant version of Ire1-HA (YKY006).

#### 4-PBA cannot restore ER damage induced by DTT

Because 4-PBA attenuated activity of bZIP-Ire1, which lacks the misfolded protein-sensing luminal domain, we questioned the idea that 4-PBA suppresses the UPR by restoring protein-folding capacity of the ER. We thus investigated protein-folding status in the ER of yeast cells in the presence and absence of 4-PBA.

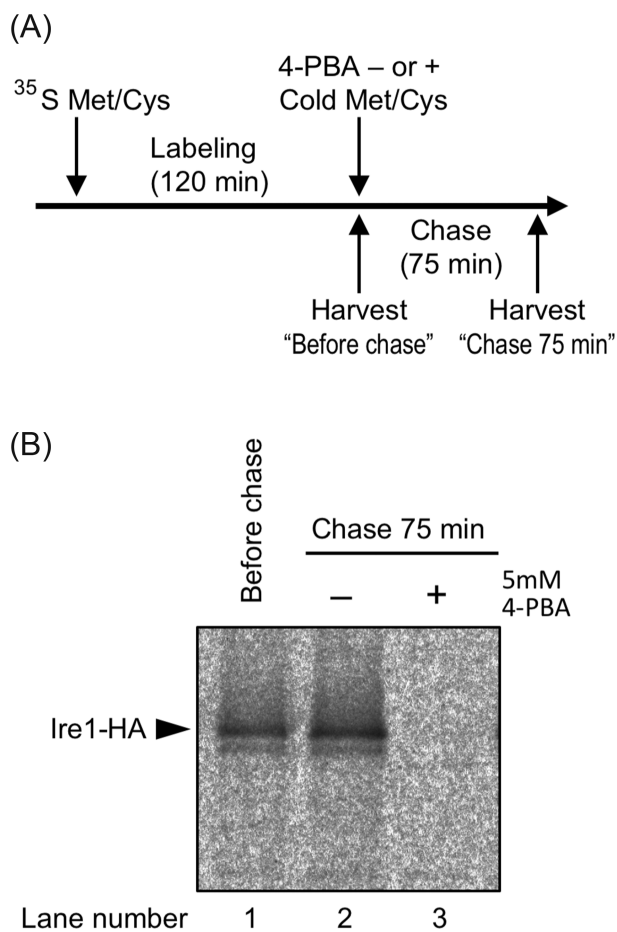
In the experiment shown in Fig. 2, we cultured cells with or without DTT and/or 4-PBA, lysed them in the presence of the mild detergent Triton X-100. The total lysate samples were then fractionated by centrifugation. Figure 2A shows that in the absence of 4-PBA, DTT treatment modestly increased the amount of BiP in the total lysate samples, indicating an induction of the UPR (compare lane 2 with 1). Importantly, similarly to our previous observation (Promlek et al. 2011), the cellular DTT treatment caused a more drastic increment of the BiP level in the pellet samples (Fig. 2B, compare lane 2 with 1). This is presumably because in yeast ER, DTT leads to the formation of protein aggregates that incorporate BiP. Since 4-PBA attenuates DTT-induced UPR (Fig. 1A and B), it suppressed the increase in BiP levels by cellular DTT treatment in the total lysate samples (Fig. 2A, compare lane 4 with 2). However, 4-PBA did not appear to affect the BiP level in the pellet samples (Fig. 2B, compare lane 3 with 1 and lane 4 with 2). This observation indicates that 4-PBA does not diminish protein aggregation in the ER of non-stressed cells and of DTT-treated cells. We then performed a similar experiment using an *ire1Δ* strain, and found that not only DTT but also 4-PBA enhances BiP sedimentation in this strain (Fig. S1, Supporting Information).

Because a mutant form of GFP, which is named as eroGFP, has an N-terminal ER-translocation signal and a C-terminal ER-retention signal (His-Asp-Glu-Leu), it is located in the ER (Merk-

samer, Trusina and Papa 2008). Moreover, eroGFP changes its excitation spectrum upon formation of a single intramolecular disulfide bond (Merkamer, Trusina and Papa 2008). Using a fluorescent microscope having two different excitation-light filters, we observed yeast cells that produce eroGFP (Fig. 3). As expected, treatment of cells with DTT changed the ratio of fluorescent intensity of eroGFP subjected to two different excitation wavelengths (compare column 2 with 1), indicating an impaired disulfide-bond formation of eroGFP. Here we showed that this ratio was not affected by 4-PBA (compare column 3 with 1 and column 4 with 2). This observation indicates that 4-PBA does not restore the ability of the ER to form protein disulfide bonds, which is the so-called oxidative protein folding and is an important part in the maturation of ER client proteins.

#### 4-PBA promotes degradation of Ire1 in yeast cells

To explore the mechanism by which 4-PBA attenuates the UPR, we next measured the cellular amount of Ire1, which is a key factor for the HAC1-mRNA splicing and the UPR induction. In the experiment shown in Fig. 4A, yeast cells that produce C-terminally HA-tagged Ire1 (Ire1-HA; Kimata et al. 2003) under the control of the IRE1 authentic promoter were assessed for their cellular Ire1-HA level through anti-HA western blot analysis of their cell lysates. We then found that cellular treatment with DTT slightly induced Ire1-HA (Fig. 4A; compare lane 2 with 1). Moreover, and importantly, 4-PBA treatment severely decreased the cellular Ire1-HA level both in the presence and absence of DTT (Fig. 4A; compare lane 3 with 1 and lane 4 with 2). Figure 4A also shows that the cellular amount of a cytosolic Pgk1 was almost unaffected by DTT or 4-PBA. Using the qRT-PCR technique

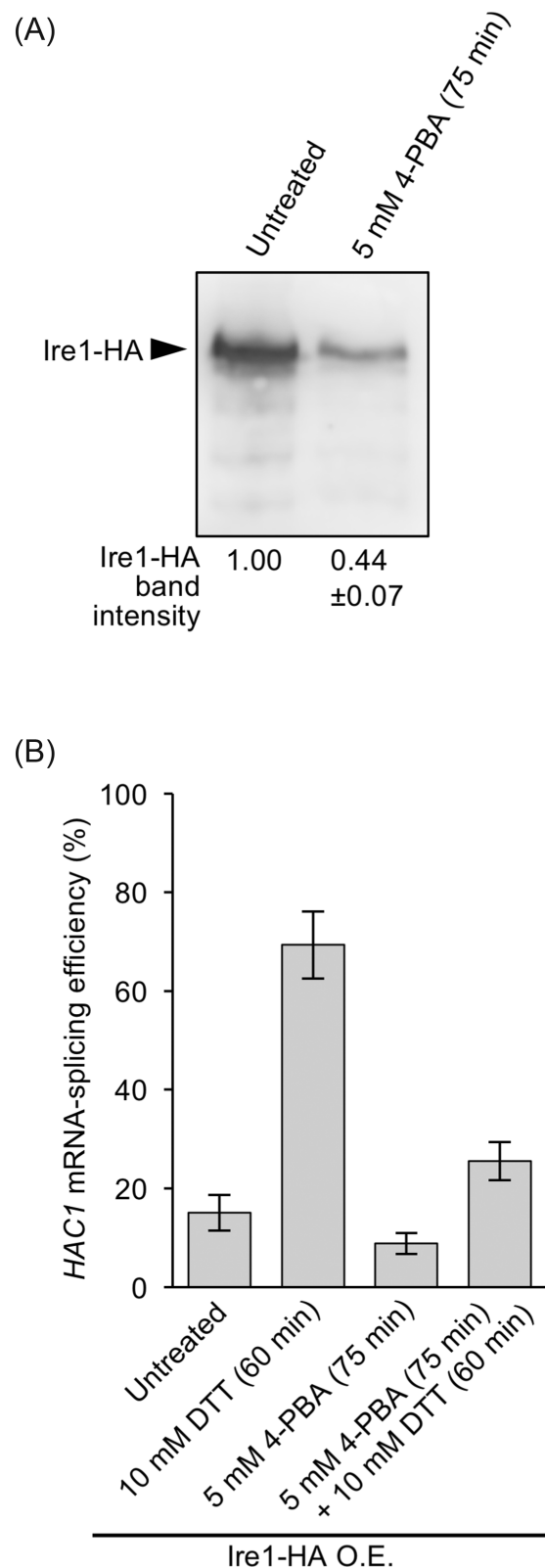


**Figure 5.** 4-PBA promotes the degradation of Ire1. (A) For the  $^{35}\text{S}$  methionine/cysteine (Met/Cys) pulse label-chase experiments, cells highly producing Ire1-HA (TKY005) were cultured in SD medium as illustrated here. (B) Harvested cells from 5-ml cultures were then lysed and subjected to anti-HA immunoprecipitation, the products of which were analyzed using SDS-DTT PAGE and autoradiography.

(Fig. 4B), we observed that 4-PBA did not appear to considerably decrease the mRNA expression level of the *IRE1* gene. As shown in Fig. 4C, 4-PBA also decreased the cellular amount of the HA-tagged version of bZIP-Ire1, albeit modestly.

We next monitored the stability of Ire1-HA in yeast cells through the  $^{35}\text{S}$  methionine/cysteine pulse labeling and chase experiment. In the experiments shown in Fig. 5, yeast cells carrying a multicopy ( $2\mu$ ) plasmid vector fused with the HA-tagged version of the *IRE1* gene (*IRE1*-HA) were labeled with  $^{35}\text{S}$  methionine/cysteine for 2 h. We then added or did not add 4-PBA into the labeled cultures simultaneously with the addition of non-labeled methionine and cysteine, which started the chase period (Fig. 5A). According to the data shown in Fig. 5B, Ire1-HA signal was almost undetectable after a 75-min chase in the presence of 4-PBA (lane 3), whereas Ire1-HA appeared to be quite stable in untreated cells (compare lane 2 with 1). This finding indicates that 4-PBA dramatically accelerates the degradation of Ire1-HA.

ER-resident transmembrane proteins are often digested through their ubiquitination and proteasomal degradation. This process is named the ER-associated degradation (ERAD), for which Hrd1 and Doa10 serve as the ubiquitin ligases. As shown in Fig. S2 (Supporting Information), 4-PBA drastically reduced the amount of Ire1-HA even in *hrd1* $\Delta$ *doa10* $\Delta$  cells. This



**Figure 6.** Ire1 overproduction mitigates the effect of 4-PBA to suppress the UPR. (A) Cells highly producing Ire1-HA (TKY005) were checked for the cellular Ire1-HA level as done in Fig. 4A. (B) The TKY005 cells were cultured as indicated and checked for the *HAC1*-mRNA splicing as done in Fig. 1A and B.

observation indicates that the ERAD is not involved in the degradation of Ire1 promoted by 4-PBA.

If 4-PBA attenuates the UPR via reduction of the cellular level of Ire1, overproduction of Ire1 may, at least partly, suppresses the effect of 4-PBA to repress the UPR. In order to validate this idea, we again employed yeast cells carrying the 2 $\mu$ -plasmid-borne IRE1-HA gene. The cellular level of overproduced Ire1-HA was decreased by cellular treatment with 4-PBA (Fig. 6A). As shown in Fig. 6B, overproduction of Ire1-HA per se slightly caused the HAC1 mRNA splicing (column 1), which was enhanced by cellular treatment with DTT (column 2), and 4-PBA lowered, but did not completely abolish, the level of HAC1 mRNA splicing both in the presence or absence of DTT (compare column 3 to 1 and column 4 to 2). Importantly and as expected, the UPR was induced even in the presence of 4-PBA, although weakly (Fig. 6B, compare column 4 to 3).

## DISCUSSION

According to *in vitro* experiments presented by Kubota *et al.* (2006), 4-PBA has an ability to function as a chemical chaperone to prevent the aggregation of denatured proteins. As mentioned in the Introduction section, this is presumably because of the chemical property of 4-PBA, a water-soluble molecule albeit with a hydrophobic moiety. Moreover, 4-PBA suppresses cellular responses against the accumulation of misfolded proteins in the ER, such as the UPR, in both yeast and mammalian cells (Kubota *et al.* 2006; Ozcan *et al.* 2006; Pineau *et al.* 2009; Le *et al.* 2016). These observations led to an idea that under some stressing conditions, 4-PBA may work as a chemical chaperone *in vivo* with an ability to totally restore the protein-folding status in the ER. 4-PBA is thus expected to have a therapeutic potential (Kolb *et al.* 2015; Ranga Rao *et al.* 2018) because misfolding and aggregation of ER client proteins are linked to numerous human diseases (Wang and Kaufman 2016). This theory may lead to an expansion of the clinical usage of 4-PBA, which is already used to alleviate the urea cycle disorder.

However, here we show that the suppression of the UPR by 4-PBA is not due to restoration of the ER protein-folding status at least in the case of yeast cells. While cells carrying bZIP-Ire1 evokes the UPR independently of the accumulation of misfolded proteins in the ER (Promlek *et al.* 2011), 4-PBA also suppresses the UPR in bZIP-Ire1 cells (Fig. 1D). Moreover, while 4-PBA almost completely abolishes the HAC1-mRNA splicing induced by DTT in IRE1 cells (Fig. 1A and B), it does not restore the DTT damage of ER protein folding (Figs 2 and 3). Conversely, 4-PBA seemed to worsen protein folding status in the ER of *ire1 $\Delta$*  cells (Fig. S1, Supporting Information). According to Bicknell *et al.* (2007), Ire1 is not absolutely required but assists maintenance of integrity of ER functions even in non-stressed cells. It is thus likely that, even under normal culturing conditions, ER conditions in IRE1 cells and *ire1 $\Delta$*  cells are somehow different, which explains the reason why 4-PBA facilitates BiP sedimentation not in IRE1 cells but in *ire1 $\Delta$*  cells.

According to our observations shown in Figs 4 and 5, 4-PBA induces a rapid degradation of Ire1, which possibly leads to the attenuation of the UPR shown in this study. Consistently to this idea, 4-PBA did not completely abolish the UPR in cells overproducing Ire1-HA (Fig. 6). By considering its fast degradation shown in Fig. 5, we deduce that the Ire1-HA molecules are mostly new borne in these cells treated with 4-PBA. It should be noted that, in yeast cells treated with DTT, the Ire1 molecules are carried along actin cables to form the Ire1 clusters, which shows a po-

tent HAC1-mRNA splicing activity (Ishiwata-Kimata *et al.* 2013). This insight may explain the reasons why the new-borne Ire1-HA poorly triggers the UPR strongly and why, in the experiment shown in Fig. 6, the UPR is only modest in the presence of both DTT and 4-PBA. The molecular mechanism by which 4-PBA accelerates the degradation of Ire1 remains unknown, since the cellular amount of Ire1-HA was considerably reduced by 4-PBA even in cells carrying the *hrd1 $\Delta$ doa10 $\Delta$*  mutation (Fig. S2, Supporting Information), which are believed to completely abolish the ERAD (Carvalho, Goder and Rapoport 2006).

Nevertheless, it should also be noted that, in certain cases, 4-PBA does exhibit an ability to restore the protein-folding condition in the ER. In mammalian cells, 4-PBA supports the folding and trafficking of ER client proteins, including the  $\Delta$ F508 CFTR mutant (Rubenstein, Egan and Zeitlin 1997; Lim *et al.* 2004). Moreover, 4-PBA is reported to improve the viability of IRE1-gene knockout yeast cells under the presence of tunicamycin (Kubota *et al.* 2006). Our findings presented here indicate that 4-PBA not only has these positive effects, but also attenuates the UPR in a different manner. We speculate that 4-PBA can assist folding of particular proteins, though not able to restore global protein folding status in cells, possibly because of substrate specificity of 4-PBA.

As aforementioned, since misfolding and aggregation of ER client proteins link to a wide variety of diseases, chemicals that assist protein folding and maturation in the ER are intriguing targets for drug discovery. The screening and evaluation of such drugs can be readily performed through monitoring the ER protein-folding status using reporter assays to assess the cellular level of the UPR (Iwawaki *et al.* 2004). However, this convenient methodology requires cautious interpretation because, as indicated here, the suppression of the UPR is not always due to an improvement of ER conditions.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSYP](https://academic.oup.com/femsyr/article/18/2/foy016/4858386) online.

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