



RESEARCH ARTICLE

Inhibition of IRE1 α -mediated XBP1 mRNA cleavage by XBP1 reveals a novel regulatory process during the unfolded protein response [version 1; referees: 2 approved with reservations]

Fiona Chalmers ¹, Bernadette Sweeney², Katharine Cain², Neil J. Bulleid ¹

¹Institute of Molecular, Cellular and Systems Biology, University of Glasgow, Glasgow, G12 8QQ, UK

²UCB Pharma Slough, Slough, SL1 3WE, UK

v1 First published: 05 Jun 2017, 2:36 (doi: [10.12688/wellcomeopenres.11764.1](https://doi.org/10.12688/wellcomeopenres.11764.1))
Latest published: 09 Oct 2017, 2:36 (doi: [10.12688/wellcomeopenres.11764.2](https://doi.org/10.12688/wellcomeopenres.11764.2))

Abstract

Background: The mammalian endoplasmic reticulum (ER) continuously adapts to the cellular secretory load by the activation of an unfolded protein response (UPR). This stress response results in expansion of the ER, upregulation of proteins involved in protein folding and degradation, and attenuation of protein synthesis. The response is orchestrated by three signalling pathways each activated by a specific signal transducer, either inositol requiring enzyme α (IRE1 α), double-stranded RNA-activated protein kinase-like ER kinase (PERK) or activating transcription factor 6 (ATF6). Activation of IRE1 α results in its oligomerisation, autophosphorylation and stimulation of its ribonuclease activity. The ribonuclease initiates the splicing of an intron from mRNA encoding the transcription factor, X-box binding protein 1 (XBP1), as well as degradation of specific mRNAs and microRNAs. **Methods:** To investigate the consequence of expression of exogenous XBP1, we generated a stable cell-line expressing spliced XBP1 mRNA under the control of an inducible promoter. **Results:** Following induction of expression, high levels of XBP1 protein were detected, which allowed upregulation of target genes in the absence of induction of the UPR. Remarkably under stress conditions, the expression of exogenous XBP1 repressed splicing of endogenous XBP1 mRNA without repressing the activation of PERK. **Conclusions:** These results illustrate that a feedback mechanism exists to attenuate activation of the Ire1 α ribonuclease activity in the presence of XBP1.

Open Peer Review

Referee Status: ? ?

Invited Referees

1 2

REVISED

version 2

published
09 Oct 2017

version 1

published
05 Jun 2017

?
report

?
report

- 1 **Stefan J. Marciniak** , University of Cambridge, UK
- 2 **Adam M. Benham** , Durham University, UK

Discuss this article

Comments (0)

Corresponding author: Neil J. Bulleid (neil.bulleid@glasgow.ac.uk)

Author roles: **Chalmers F:** Investigation, Validation, Writing – Original Draft Preparation; **Sweeney B:** Funding Acquisition, Project Administration; **Cain K:** Conceptualization, Funding Acquisition, Project Administration; **Bulleid NJ:** Conceptualization, Funding Acquisition, Project Administration, Supervision, Validation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

How to cite this article: Chalmers F, Sweeney B, Cain K and Bulleid NJ. **Inhibition of IRE1 α -mediated XBP1 mRNA cleavage by XBP1 reveals a novel regulatory process during the unfolded protein response [version 1; referees: 2 approved with reservations]** Wellcome Open Research 2017, 2:36 (doi: [10.12688/wellcomeopenres.11764.1](https://doi.org/10.12688/wellcomeopenres.11764.1))

Copyright: © 2017 Chalmers F *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution Licence](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Grant information: The work was supported by the Wellcome Trust [103720]; and Biotechnology and Biological Sciences Research Council [BB/K004395/1].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

First published: 05 Jun 2017, 2:36 (doi: [10.12688/wellcomeopenres.11764.1](https://doi.org/10.12688/wellcomeopenres.11764.1))

Introduction

The endoplasmic reticulum (ER) is the site of protein folding and post-translational modification of secreted and transmembrane proteins¹. Under stress conditions such as glucose starvation or a viral infection, the folding capacity of the ER can become compromised, leading to a potentially lethal build-up of unfolded or misfolded proteins². Protein folding homeostasis can be restored by triggering of a stress response called the unfolded protein response (UPR)³. This complex and tightly-regulated process has downstream effects that enable the ER to adapt to stress conditions, and if this pro-survival strategy does not successfully restore ER homeostasis then pro-apoptotic signalling is induced⁴.

The mammalian UPR is formed from three distinct but overlapping signalling branches, each governed by an initial effector protein localised to the ER membrane. These proteins are activating transcription factor 6 (ATF6), protein kinase-like ER kinase (PERK) and inositol requiring enzyme α (IRE1 α), and are activated in the presence of a build-up of incorrectly folded proteins⁵. Of these three mammalian UPR effectors, IRE1 α is the most conserved with its yeast homolog being solely responsible for the UPR in lower eukaryotes⁶. The activation of its cytosolic endoribonuclease (RNase) domain enables processing of unspliced X-box binding protein 1 (XBP1) mRNA (XBP1u). Spliced transcripts (XBP1s) are translated into the protein XBP1^S, a transcription factor that upregulates the expression of proteins involved in ER protein folding, ER associated degradation (ERAD) and lipid biogenesis as part of a concerted effort to increase the capacity of the ER to cope with unfolded proteins.

In addition to its RNase domain, IRE1 α also contains a cytosolic kinase domain and a luminal domain that senses ER stress, and these domains are connected by a single transmembrane domain⁷. Upon activation, IRE1 α forms dimers⁸, via 'face-to-face' interactions⁹. The 'face-to-face' dimer displays no RNase activity and represents an early stage in IRE1 α activation; its main purpose is to bring the kinase domains into proximity to enable transautophosphorylation. Phosphorylation induces a change in structure into a 'back-to-back' dimer⁹, which brings the RNase domains into direct contact, forming a functional RNase active site capable of splicing XBP1u.

Activated IRE1 α is also able to digest mRNAs¹⁰ and miRNAs¹¹ during a process termed Regulated IRE1 α Dependent Decay (RIDD). It has been suggested that the specificity of IRE1 α changes during the UPR, initially cleaving XBP1u, but during prolonged stress switching to the cleavage of mRNA coding for proteins upregulated during the UPR. The consequence is an exacerbation of the stress leading to apoptosis¹². In addition, the cleavage of miRNAs responsible for the downregulation of caspase-2 results in elevated levels of this protease and induction of apoptosis through the BAX/BAK-dependent pathway¹¹. What regulates this switch in specificity is unknown, but could be related to subtle changes in IRE1 α phosphorylation status, or conformation or interaction with IRE1 α regulators¹³.

Given the potential for IRE1 α to activate proapoptotic factors during prolonged ER stress, it is important to understand how IRE1 α activity is attenuated. Previous studies indicate that this attenuation may be the result of multiple mechanisms to reduce IRE1 α protein, reverse oligomerisation or alter phosphorylation status. For example, IRE1 α transcripts can be degraded by RIDD¹⁴ and activated IRE1 α dimers can be dephosphorylated by the phosphatase PP2C¹⁵. In addition, the oxidation of thiols within the IRE1 α luminal domain occurs during activation, a modification that is reversed during IRE1 α attenuation. This mechanism of attenuation is dependent upon oxidoreductase activity provided by P5, a member of the protein disulfide isomerase family¹⁶. Finally the depletion of XBP1^S can be facilitated by XBP1^U, the protein translated from XBP1u transcripts, and involves the binding of XBP1^U to XBP1^S and subsequent trafficking to the 26S proteasome for degradation¹⁷.

In order to explore the regulatory mechanisms of IRE1 α , we investigated the impact of high levels of expression of XBP1^S on the activity of IRE1 α . Our results show that an abundance of XBP1^S represses endogenous XBP1 splicing during unstressed and stress conditions. This repression may represent a regulatory mechanism, where persistent ER stress attenuates IRE1 α RNase activity at least towards XBP1 mRNA.

Materials and methods

Generation of stable cell lines

CHO-S X was generated by transfecting 4 μ g of pTetOne vector (Clontech), containing cDNA for the human XBP1s sequence, into CHO-S cells (Life Technologies), co-transfected with 200ng of a linear selectable marker for puromycin (a vector:marker ratio of 20:1), with 4.2 μ l of the transfection reagent NovaCHOice (Novagen). Transfected cells and untransfected control cells were grown in a 6 cm diameter dish in adherent culture, and after 24 h of growth were trypsinised and 1/10 of the cells were transferred to a 15 cm dish and grown in 20 ml medium containing 12.5 μ g/ml puromycin. The transfected cells were grown for approximately 10 days, refreshing the selection medium every 3–4 days. Colonies were identified and removed from the dish using trypsin-soaked cloning discs and transferred into the wells of a 12 well plate, with one colony per well. The clones were grown under selection for another 3–5 days until the well was confluent, then the surviving clones were transferred into T25 flasks and later T75 flasks. To generate the CHO-S XB cell line, CHO-S X cells were transfected with a BFP construct¹⁸ using the same method as described above. The construct contained a G418 resistance gene, and therefore the linear selectable marker was not required. Transfected cells were maintained under the dual selection of both 12.5 μ g/mL puromycin and 2 mg/mL G418 (Promega) to maintain the BFP construct. Successful integration of the gene of interest was confirmed by western blotting.

Maintenance of cell lines

CHO-S and CHO-S XB cells were grown in DMEM supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, and

non-essential amino acids at a working concentration of 10 μ M for each amino acid (Gibco). Cells were grown as an adherent culture and split every 3–4 days using a standard trypsin protocol.

XBP1 splicing assay

RNA was extracted from stress treated cells using Trizol Reagent (Ambion), following the manufacturer's recommended protocol. First strand cDNA was synthesised using Superscript II Reverse Transcriptase (Invitrogen) with oligo dTs (Invitrogen), according to the manufacturer's specifications. cDNA for endogenous XBP1 was amplified using primers designed using CLC Genomics Workbench (v6) (RRID:SCR_011853) to be specific to the Chinese hamster XBP1 sequence, (5'-CGCTTGGGAATGGATG-3' and 5'-CAGGGTCCAACCTGTCC-3'; Sigma-Aldrich). The PCR reaction yielded a 247 bp fragment for XBP1u and a 215 bp fragment for XBP1s, plus a hybrid band of approximately 280 bp. Both endogenous and exogenous XBP1 were amplified simultaneously with a second, less specific set of primers, which can anneal to either the Chinese hamster or the human XBP1 sequence, 5'-ACAGCGCTTGGGGATGGATG-3' and 5'-TGACTGGGTC-CAAGTTGTCC-3' (Sigma-Aldrich). PCR using these primers yielded the same fragments as the previous primer set, but with the addition of a fragment of 221 bp for exogenous XBP1s. PCR was performed with Accuzyme DNA polymerase (Bioline) with an initial melting step of 95°C for 5 min; then 35 cycles of 95°C for 45 s, an annealing step for 45 s, and 72°C for 45 s; followed by a final elongation step of 72°C for 10 min. The endogenous only primers used an annealing temperature of 60°C and the exogenous/endogenous primers used 62°C.

Cell lysis

After removing culture medium from the 6 cm diameter dish, the cells were washed with 20 mM NEM in PBS for 10 min. This was removed and 120 μ l lysis buffer (50 mM Tris-HCl buffer containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 4mM NaF) was added to the monolayer and the cells were scraped into the buffer. This suspension was left on ice for 10 min, centrifuged at maximum speed for 10 min and the supernatant was extracted.

SDS PAGE

Crude lysates were mixed with 0.2 M Tris-HCl (pH 6.8) containing 10% (w/v) SDS, 20% (v/v) glycerol and 0.05% (w/v) bromophenol blue (sample buffer) in a 4:1 ratio of lysate to sample buffer. Dithiothreitol (DTT) was added as a reducing agent at a working concentration of 20 mM. Polyacrylamide gels were loaded with 20–30 μ l of this sample mixture and run at 20 mA per gel.

Western blot

After separation, the samples were transferred to a nitrocellulose membrane (GE Healthcare) by wet transfer for 1 h at 250 mA using 25 mM Tris-HCl containing 200 mM glycine, 3.5 mM SDS and 20% (v/v) methanol. The blots were blocked in 5% (w/v) non-fat milk powder (Marvel) in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 0.1% (v/v) Tween (TBST) for 1 h. Primary antibodies were diluted in TBST and incubated overnight. Washes were performed three times for 10 min in TBST. Secondary antibodies

were diluted in TBST and incubated for 1 h, and the blots shielded from light throughout the incubation. Blots were developed using the Odyssey SA scanner (Licor). The following primary antibodies were used: rabbit polyclonal anti-PDI¹⁹, 1:500; rabbit polyclonal anti-XBP1^S (Biolegend, Cat# 619502, RRID:AB_315907), 1:500; rabbit monoclonal anti-PERK, (Cell Signalling, Cat# 3192 RRID:AB_2095847), 1:1000; mouse monoclonal anti-GAPDH, (Ambion, Cat# AM4300, RRID:AB_437392), 1:10000; rabbit polyclonal anti-actin, (Sigma-Aldrich Cat# A2103, RRID:AB_476694), 1:500. The following secondary antibodies were used: donkey polyclonal anti-mouse 680RD, (LI-COR Biosciences Cat# 926-68072, RRID:AB_10953628) 1:10000; donkey polyclonal anti-mouse 800CW (LI-COR Biosciences Cat# 926-32212, RRID:AB_621847), 1:10000; donkey polyclonal anti-rabbit 680RD, (LI-COR Biosciences Cat# 926-68073, RRID:AB_10954442), 1:10000; donkey polyclonal anti-rabbit 800CW (LI-COR Biosciences Cat# 926-32213, RRID:AB_621848), 1:10000.

ER Tracker treatment

ER Tracker Green BODIPY FL Glibenclamide (Molecular Probes) was dissolved in DMSO to a 1 mM stock concentration. Treated cells were stained with 250 nM ER Tracker in Hank's Balanced Salt Solution (HBSS) for 30 min, then trypsinised and resuspended in DMEM.

ER stress treatments

Dithiothreitol (DTT) (Melford Labs) was prepared as 1 M stock in water and used at a 2.5 mM working concentration. Thapsigargin (Sigma-Aldrich) was prepared as a 2 mM stock in DMSO and used at 4 μ M. Tunicamycin (Sigma-Aldrich) was prepared as a 10 mg/mL stock in DMSO and used at 10 μ g/mL. PERK inhibitor (Tocris) was prepared as a 10mM stock in DMSO and used at 2.5 μ M. Treatments were added for 3 h to cells that had been pretreated with doxycycline for 48 h.

Flow cytometry

Cells were washed once in HBSS then run on a FACS Canto II flow cytometer (BD Biosciences) in HBSS. ER Tracker Green has an excitation at 504 nm and emission at 511 nm, which can be detected using the FITC filter on the flow cytometer. The data obtained was analysed using [Flowing Software v2.5.1](#) (Turku Bioimaging).

Results

To investigate the consequence of overexpression of human XBP1s in CHO-S cells, we created a stable cell-line expressing XBP1s mRNA under the control of a doxycycline inducible promoter. No XBP1^S was detected unless doxycycline was included in the medium ([Figure 1](#)). Such tight regulation of expression allowed us to evaluate the effect of overexpression of XBP1s in the same cell line simply by culturing cells in the presence or absence of doxycycline.

To study the activation of IRE1 α RNase activity, we assessed the cleavage of XBP1u mRNA before and after induction of human XBP1s expression, using an RT-PCR assay²⁰. By designing primers that flank the XBP1 spliced intron, cDNA derived from XBP1s and XBP1u transcripts can be amplified by PCR and distinguished from each other by a subtle, 26bp difference in product size when run on

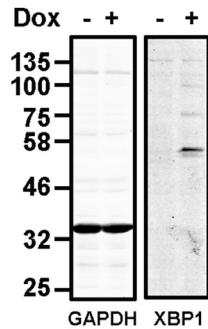


Figure 1. Induction of XBP1s expression in CHO-S XB cells. Western blot of lysates from CHO-S XB cells either uninduced or induced with doxycycline (Dox) for 3 days, probed with anti-XBP1s and anti-GAPDH as indicated. The blot is representative of the results obtained from three separate experiments.

an agarose gel. This assay is also known to generate a third PCR product, shown diagrammatically (Figure 2A), which is thought to be a hybrid double-stranded cDNA product consisting of one strand XBP1s and one strand XBP1u. This hybrid product migrates above double-stranded XBP1u on an agarose gel due to its bulkier structure²⁰. Two sets of primers were designed for the assay: the first set was designed to only anneal to the endogenous hamster sequence of XBP1, and these primers were used to quantify only endogenous XBP1s and XBP1u. The second set was designed to bind to both forms of endogenous CHO XBP1, and also to the exogenous human XBP1s transcript, allowing visualisation of all forms of XBP1 present in the cell.

In the absence of cell-stress, the endogenous XBP1 mRNA in CHO-S cells was present as a mixture of unspliced and hybrid forms (Figures 2B and D). This result is consistent with a basal

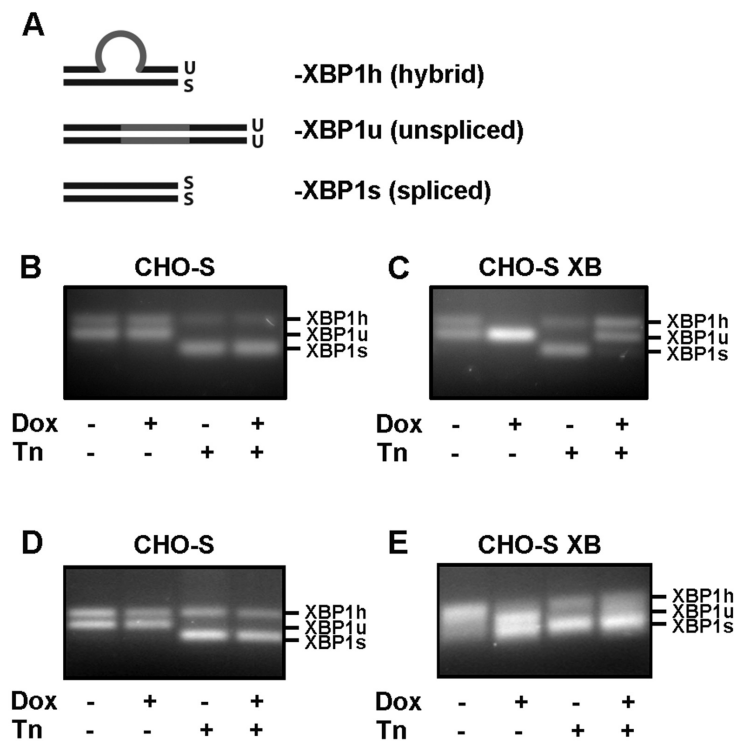


Figure 2. Consequence of XBP1s expression on XBP1u splicing in cell lines following endoplasmic reticulum stress. (A) Schematic of the three possible cDNA products generated by primers that flank the XBP1 spliced intron (indicated in grey). Single stranded cDNA can anneal to either its complementary strand to generate double stranded XBP1u and XBP1s products, or generate a hybrid, XBP1h, formed from one strand XBP1u and one strand XBP1s. Of these three PCR products, XBP1s migrates the furthest on an agarose gel, followed by XBP1u and then by XBP1h, leading to the appearance of three distinct DNA bands. Adapted from 20. (B–E) XBP1 splice assay of cDNA extracted from either (B,D) CHO-S or (C,E) CHO-S XB cells treated with doxycycline (Dox; for 3 days) and/or tunicamycin (Tn; for 3 h) as indicated. PCR reactions contained primers that anneal only to the endogenous CHO XBP1 sequence (B,C) or primers that anneal to both the endogenous sequence and the exogenous XBP1s transcripts (D,E). The experiment was performed at least three times, data from a representative experiment is shown.

level of UPR signalling reported to be active under normal physiological conditions²¹. As expected, there was no change in the splicing pattern following treatment with doxycycline, indicating that this chemical alone does not induce the UPR. Following treatment with the ER stress inducer tunicamycin, all of the XBP1 mRNA was converted to either the spliced or hybrid form indicative of a strong UPR (Figures 2B and D). The splicing pattern of endogenous XBP1 mRNA in CHO-S XB cells in the absence of doxycycline, with or without cell stress was similar to that in CHO-S cells (Figure 2C). However, in the absence of cell stress incubation with doxycycline to induce XBP1s expression prevented any splicing of endogenous XBP1 mRNA, as evidenced by the absence of the hybrid form. Doxycycline induced expression of exogenous XBP1s, as seen by the presence of XBP1s when primers recognising both the human and hamster XBP1 were used in the assay (Figure 2E). We consistently observed an increase in expression of XBP1u following induction of XBP1s expression, indicating the upregulation of XBP1u expression by XBP1s, as shown previously^{20,22}. Under conditions of ER stress XBP1 mRNA was efficiently spliced in the absence of doxycycline, but this splicing was dramatically repressed after doxycycline treatment (Figure 2C). This result indicates that XBP1 splicing by IRE1 α is largely prevented in cells overexpressing XBP1s.

To determine whether there was a correlation between the induction of expression of exogenous XBP1s and the repression of IRE1 α cleavage of endogenous XBP1 mRNA, we titrated the amount of added doxycycline to induce increasing amounts of exogenous XBP1s. The effect on cleavage of endogenous XBP1 mRNA became apparent after treating with 50 ng/ml of doxycycline both in the absence or presence of tunicamycin-induced ER stress (Figures 3A and B). The effect increased with increasing concentrations of doxycycline with the greatest repression being most apparent at 1000 ng/ml in the presence of tunicamycin. When

the presence of exogenously expressed XBP1s was evaluated using primers that amplify endogenous and exogenous XBP1, a clear increase in the XBP1s signal was observed at 50 ng/ml, which increased in intensity up to the highest concentration of doxycycline used (Figure 3C). These results show a clear correlation between XBP1s expression and the repression of IRE1 α -mediated cleavage of endogenous XBP1.

The expression of exogenous XBP1s should lead to the upregulation of a number of proteins that are known to alleviate ER stress. Hence, the expression of XBP1s could prevent or suppress the tunicamycin-mediated activation of IRE1 α , thereby repressing its RNase activity. To determine the consequence of XBP1s expression on ER expansion, we stained cells with ER Tracker, a dye that binds to potassium channels prominent at the ER membrane²³. Green fluorescence per cell was seen to increase following doxycycline treatment in CHO-S XB but not CHO-S cells, as quantified by FACS analysis (Figure 4A). This result indicates that the expression of exogenous XBP1 does indeed cause an expansion in the ER, as seen previously when XBP1s is overexpressed in CHO cells²⁴. To determine whether XBP1s expression leads to a suppression of other branches of the UPR, we evaluated the activation of PERK, indicated by autophosphorylation. PERK phosphorylation was assayed by a shift in electrophoretic mobility to a slower migrating form after UPR induction, exemplified after treatment with DTT or the presence of a PERK kinase inhibitor²⁵ (Figure 4B). There was no effect on PERK phosphorylation after treatment with a variety of UPR inducers (DTT, thapsigargin or tunicamycin) in the presence or absence of doxycycline in CHO-S XB cells. This result indicates that there is still a robust UPR activated following treatment with tunicamycin in cells overexpressing XBP1s as judged by PERK phosphorylation. This result suggests that the overexpression of XBP1s does not suppress the ability of UPR activators to induce a UPR.

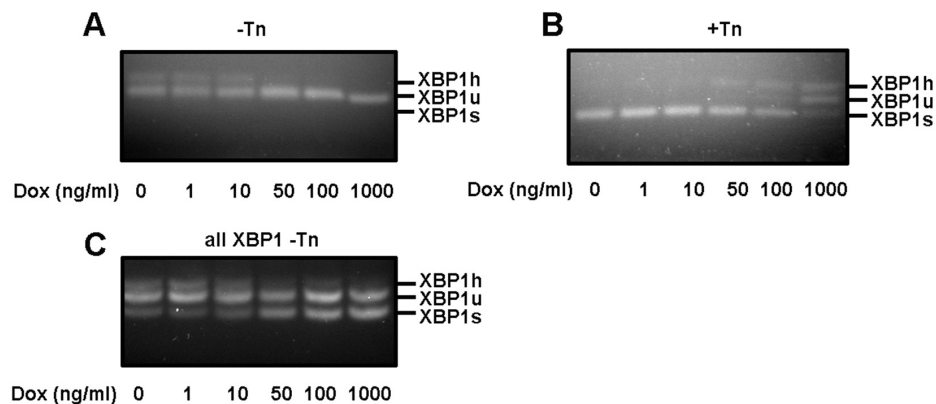


Figure 3. Inhibition of XBP1 splicing correlates with the amount of XBP1s. XBP1 splice assay of cDNA from CHO-S XB cells induced for 3 days with a range of doxycycline (Dox) concentrations, either not treated with tunicamycin (Tn) (A,C), or treated with 10 μ g/mL Tn for 3 h. Primers specific for endogenous XBP1 (A,B) or endogenous and exogenous XBP1 (C) were used. The experiment was performed twice, data from a representative experiment is shown.

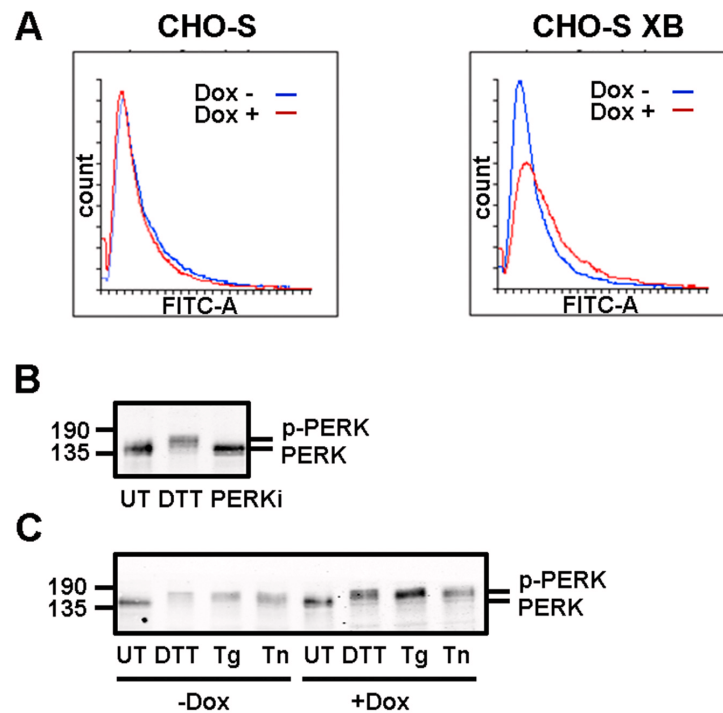


Figure 4. PERK activation is unchanged by overexpression of XBP1S. (A) Flow cytometry analysis of fluorescence from CHO-S and CHO-S XB cells stained with fluorescent ER Tracker dye. Samples were either treated with doxycycline (Dox) for 3 days (red) or left untreated (blue). (B) Western blot of lysates from CHO-S XB cells that were either untreated (UT), treated with a reducing agent (DTT) or with an inhibitor of PERK kinase activity (PERKi). Blots were probed with anti-PERK to display the extent of PERK phosphorylation. (C) Anti-PERK western blot of CHO-S XB cells induced with Dox and subsequently treated with DTT, thapsigargin (Tg) or tunicamycin (Tn). Each experiment was performed once.

Discussion

Activation of the IRE1 α branch of the UPR can lead to a variety of outcomes based on regulation of its RNase activity²⁶. This activity is tightly controlled by a number of mechanisms, including the transcript and protein levels of IRE1 α , changes to its quaternary structure and by its phosphorylation and redox state. Our results indicate an additional mechanism of IRE1 α feedback regulation involving XBP1s, which is able to repress RNase activity towards XBP1u. This regulation was revealed upon overexpression of XBP1s and occurred in the absence and presence of ER stress.

Overexpression of XBP1s leads to high levels of expression of XBP1^s protein, to the expansion of the ER and an increased expression of secreted proteins^{24,27}. Preconditioning the ER to stress by XBP1s expression could prevent IRE1 α activation, thereby repressing splicing of endogenous XBP1u. However, we showed that robust activation of PERK still occurs in cells overexpressing XBP1s upon ER stress. As the mechanism for PERK and Ire1 α activation requires BiP dissociation it seems unlikely that the absence of Ire1 α activation is due to increased levels of BiP. Hence, activation of IRE1 α should occur even in the presence of XBP1s. The repression of IRE1 α ability to splice XBP1u is, therefore, most likely to occur downstream of its activation during stress conditions.

Activation of IRE1 α leads to its phosphorylation, which has been shown to promote dimerisation of its cytosolic domain²⁸. This suggests that phosphorylation activates IRE1 α , whereas a phosphatase could be responsible for attenuating IRE1 α . One phosphatase, PP2Ce, has been suggested to perform this role¹⁵; however, the promoter for this gene does not display the ERSE, ERSE-II or UPRE consensus sequences characteristic of genes upregulated by XBP1s^{29,30}. Also, it has been shown that hyperphosphorylation rather than dephosphorylation of yeast IRE1 is required to deactivate this protein³¹. Nevertheless, the possibility remains that an XBP1s-inducible phosphatase could attenuate the activity of IRE1 α during prolonged induction of the UPR.

Alternatively, XBP1^s could block the initial phosphorylation and dimerisation of IRE1 α in order to reduce the overall intensity of IRE1 α signalling. In support of this hypothesis, it was reported that XBP1^s works in complex with Sec63 and BiP to negatively regulate IRE1 α autophosphorylation³². A mouse Sec63 knockout cell line was shown to constitutively activate IRE1 α phosphorylation, regardless of the presence of ER stress. Intriguingly, this study revealed that the overexpression of XBP1^s in the Sec63 knockout cell line was able to abolish the activation of IRE1 α almost entirely, even in the presence of tunicamycin, indicating that Sec63 and XBP1^s work in concert to regulate IRE1 α phosphorylation.

However, this study did not examine the effect of XBP1^s overexpression on IRE1 α activation in a cell line with physiological levels of Sec63; circumstances that would be closer to the conditions used in the results reported here.

The abundance of IRE1 α can be modulated by proteasomal degradation initiated by ubiquitination by the E3 ubiquitin ligase synoviolin (SYVN1), otherwise known as the ERAD component HRD1³³. The ubiquitination of BiP-bound IRE1 α monomers by SYVN1 leads to its dislocation from the ER and degradation by the proteasome. Under normal physiological conditions, BiP-bound IRE1 α is continually degraded by ERAD, but the detachment of BiP allows for IRE1 α to bypass interaction with SYVN1 and undergo accumulation and activation³⁴. Like other components of ERAD, SYVN1 is upregulated by XBP1s so it can be assumed that CHO-S XB would display high levels of SYVN1. This could lead to a reduction in IRE1 α protein in XBP1s overexpressing cells; however, as only BiP-bound IRE1 α is targeted for ERAD it is only this inactive form that would be affected by XBP1s upregulation, and not activated dimers. However, there is precedent for a reduction in total IRE1 α unrelated to proteasomal degradation in the presence of stress. Heat shock treatment was shown to deplete IRE1 α in a range of mammalian cell lines in a manner that could not be blocked by a proteasome inhibitor³⁵. The UPR was activated in these cells, indicated by the presence of IRE1 α phosphorylation and other UPR hallmarks, but the specific mechanism for the degradation of IRE1 α could not be clarified, and was attributed to an unknown method of suppressing extreme UPR signalling. It is possible that this mechanism could be mediated via XBP1s.

While we have noted here that XBP1s overexpression repressed XBP1u splicing activity, we have not investigated whether there is any suppression or even activation of activity towards other RNA

substrates. It has been shown previously that overexpression of XBP1s had no effect on cell viability under non-stress conditions¹². Hence, under these conditions there was no suppression or activation of IRE1 α RNase activity towards substrates other than XBP1u. It remains to be determined whether under stress conditions and in the presence of excess XBP1s, repression of IRE1 α RNase activity extends to all mRNAs not just XBP1u.

Dysregulated IRE1 α is a known contributing factor to a number of diseases, including multiple myeloma³⁶, epithelial cancers³⁷, Parkinson's disease³⁸ and inflammatory bowel disease³⁹. Hence, increased knowledge of the regulatory mechanisms controlling IRE1 α activity will help in understanding the pathogenesis of these diseases, as well as improving any therapeutic intervention.

Data availability

The uncropped western blots and the FACS files can be found on the Open Source Framework (DOI: [10.17605/OSF.IO/BGCDE](https://doi.org/10.17605/OSF.IO/BGCDE);⁴⁰)

Competing interests

No competing interests were disclosed.

Grant information

The work was supported by the Wellcome Trust [103720]; and Biotechnology and Biological Sciences Research Council [BB/K004395/1].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Marie Anne Pringle for support during this work.

References

- Braakman I, Bulleid NJ: **Protein folding and modification in the mammalian endoplasmic reticulum.** *Annu Rev Biochem.* 2011; **80**: 71–99.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kaufman RJ, Scheuner D, Schröder M, *et al.*: **The unfolded protein response in nutrient sensing and differentiation.** *Nat Rev Mol Cell Biol.* 2002; **3**(6): 411–21.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Travers KJ, Patil CK, Wodicka L, *et al.*: **Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation.** *Cell.* 2000; **101**(3): 249–58.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Walter P, Ron D: **The unfolded protein response: from stress pathway to homeostatic regulation.** *Science.* 2011; **334**(6059): 1081–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kebache S, Cardin E, Nguyễn DT, *et al.*: **Nck-1 antagonizes the endoplasmic reticulum stress-induced inhibition of translation.** *J Biol Chem.* 2004; **279**(10): 9662–71.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ron D, Walter P: **Signal integration in the endoplasmic reticulum unfolded protein response.** *Nat Rev Mol Cell Biol.* 2007; **8**(7): 519–29.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Sidrauski C, Walter P: **The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response.** *Cell.* 1997; **90**(6): 1031–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
- van Anken E, Pincus D, Coyle S, *et al.*: **Specificity in endoplasmic reticulum-stress signaling in yeast entails a step-wise engagement of HAC1 mRNA to clusters of the stress sensor Ire1.** *eLife.* 2014; **3**: e05031.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Joshi A, Newbatt Y, McAndrew PC, *et al.*: **Molecular mechanisms of human IRE1 activation through dimerization and ligand binding.** *Oncotarget.* 2015; **6**(15): 13019–35.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hollien J, Lin JH, Li H, *et al.*: **Regulated Ire1-dependent decay of messenger RNAs in mammalian cells.** *J Cell Biol.* 2009; **186**(3): 323–31.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Upton JP, Wang L, Han D, *et al.*: **IRE1 α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2.** *Science.* 2012; **338**(6108): 818–22.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Han D, Lerner AG, Vande Walle L, *et al.*: **IRE1 α kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates.** *Cell.* 2009; **138**(3): 562–75.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Chen Y, Brandizzi F: **IRE1: ER stress sensor and cell fate executor.** *Trends Cell*

- Biol.* 2013; **23**(11): 547–55.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
14. Tirasophon W, Lee K, Callaghan B, *et al.*: **The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response.** *Genes Dev.* 2000; **14**(21): 2725–36.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 15. Lu G, Ota A, Ren S, *et al.*: **PPM1f encodes an inositol requiring-protein 1 (IRE1) specific phosphatase that regulates the functional outcome of the ER stress response.** *Mol Metab.* 2013; **2**(4): 405–16.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 16. Eletto D, Eletto D, Dersh D, *et al.*: **Protein disulfide isomerase A6 controls the decay of IRE1 α signaling via disulfide-dependent association.** *Mol Cell.* 2014; **53**(4): 562–76.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 17. Yoshida H, Oku M, Suzuki ML, *et al.*: **pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response.** *J Cell Biol.* 2006; **172**(4): 565–75.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 18. Costantini LM, Balaban M, Markwardt ML, *et al.*: **A palette of fluorescent proteins optimized for diverse cellular environments.** *Nat Commun.* 2015; **6**: 7670.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 19. John DC, Grant ME, Bulleid NJ: **Cell-free synthesis and assembly of prolyl 4-hydroxylase: the role of the beta-subunit (PDI) in preventing misfolding and aggregation of the alpha-subunit.** *EMBO J.* 1993; **12**(4): 1587–95.
[PubMed Abstract](#) | [Free Full Text](#)
 20. Shang J, Lehrman MA: **Discordance of UPR signaling by ATF6 and Ire1p-XBP1 with levels of target transcripts.** *Biochem Biophys Res Commun.* 2004; **317**(2): 390–6.
[PubMed Abstract](#) | [Publisher Full Text](#)
 21. Wang Y, Xing P, Cui W, *et al.*: **Acute Endoplasmic Reticulum Stress-Independent Unconventional Splicing of XBP1 mRNA in the Nucleus of Mammalian Cells.** *Int J Mol Sci.* 2015; **16**(6): 13302–21.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 22. Yoshida H, Okada T, Haze K, *et al.*: **ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response.** *Mol Cell Biol.* 2000; **20**(18): 6755–67.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 23. Zunkler BJ, Wos-Maganga M, Panten U: **Fluorescence microscopy studies with a fluorescent glibenclamide derivative, a high-affinity blocker of pancreatic beta-cell ATP-sensitive K⁺ currents.** *Biochem Pharmacol.* 2004; **67**(8): 1437–44.
[PubMed Abstract](#) | [Publisher Full Text](#)
 24. Cain K, Peters S, Hailu H, *et al.*: **A CHO cell line engineered to express XBP1 and ERO1-L α has increased levels of transient protein expression.** *Biotechnol Prog.* 2013; **29**(3): 697–706.
[PubMed Abstract](#) | [Publisher Full Text](#)
 25. Axten JM, Medina JR, Feng Y, *et al.*: **Discovery of 7-methyl-5-(1-((3-(trifluoromethyl)phenyl)acetyl)-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK).** *J Med Chem.* 2012; **55**(16): 7193–207.
[Publisher Full Text](#)
 26. Ghosh R, Wang L, Wang ES, *et al.*: **Allosteric inhibition of the IRE1 α RNase preserves cell viability and function during endoplasmic reticulum stress.** *Cell.* 2014; **158**(3): 534–48.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 27. Gulis G, Simi KC, de Toledo RR, *et al.*: **Optimization of heterologous protein production in Chinese hamster ovary cells under overexpression of spliced form of human X-box binding protein.** *BMC Biotechnol.* 2014; **14**: 26.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 28. Lee KP, Dey M, Neculai D, *et al.*: **Structure of the dual enzyme ire1 reveals the basis for catalysis and regulation in nonconventional RNA splicing.** *Cell.* 2008; **132**(1): 89–100.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 29. Yamamoto K, Yoshida H, Kokame K, *et al.*: **Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II.** *J Biochem.* 2004; **136**(3): 343–50.
[PubMed Abstract](#) | [Publisher Full Text](#)
 30. Lee AH, Iwakoshi NN, Glimcher LH: **XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response.** *Mol Cell Biol.* 2003; **23**(21): 7448–59.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 31. Rubio C, Pincus D, Korennykh A, *et al.*: **Homeostatic adaptation to endoplasmic reticulum stress depends on Ire1 kinase activity.** *J Cell Biol.* 2011; **193**(1): 171–84.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 32. Fedeles SV, So JS, Shrikhande A, *et al.*: **Sec63 and Xbp1 regulate IRE1 α activity and polycystic disease severity.** *J Clin Invest.* 2015; **125**(5): 1955–67.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 33. Gao B, Lee SM, Chen A, *et al.*: **Synoviolin promotes IRE1 ubiquitination and degradation in synovial fibroblasts from mice with collagen-induced arthritis.** *EMBO Rep.* 2008; **9**(5): 480–5.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 34. Sun S, Shi G, Sha H, *et al.*: **IRE1 α is an endogenous substrate of endoplasmic-reticulum-associated degradation.** *Nat Cell Biol.* 2015; **17**(12): 1546–55.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 35. Homma T, Fujii J: **Heat stress promotes the down-regulation of IRE1 α in cells: An atypical modulation of the UPR pathway.** *Exp Cell Res.* 2016; **349**(1): 128–138.
[PubMed Abstract](#) | [Publisher Full Text](#)
 36. Chen L, Li Q, She T, *et al.*: **IRE1 α -XBP1 signaling pathway, a potential therapeutic target in multiple myeloma.** *Leuk Res.* 2016; **49**: 7–12.
[PubMed Abstract](#) | [Publisher Full Text](#)
 37. Jin C, Jin Z, Chen NZ, *et al.*: **Activation of IRE1 α -XBP1 pathway induces cell proliferation and invasion in colorectal carcinoma.** *Biochem Biophys Res Commun.* 2016; **470**(1): 75–81.
[PubMed Abstract](#) | [Publisher Full Text](#)
 38. Mercado G, Castillo V, Soto P, *et al.*: **ER stress and Parkinson's disease: Pathological inputs that converge into the secretory pathway.** *Brain Res.* 2016; **1648**(Pt B): 626–632.
[PubMed Abstract](#) | [Publisher Full Text](#)
 39. Kaser A, Martínez-Naves E, Blumberg RS: **Endoplasmic reticulum stress: implications for inflammatory bowel disease pathogenesis.** *Curr Opin Gastroenterol.* 2010; **26**(4): 318–26.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 40. Bulleid N: **"Inhibition of IRE1 α -Mediated XBP1 mRNA Cleavage by XBP1."** *Open Science Framework.* 2017.
[Data Source](#)

Open Peer Review

Current Referee Status: ? ?

Version 1

Referee Report 26 June 2017

doi:[10.21956/wellcomeopenres.12708.r23238](https://doi.org/10.21956/wellcomeopenres.12708.r23238)



Adam M. Benham 

Department of Biosciences, Durham University, Durham, UK

This is an interesting report suggesting that novel feedback mechanisms exist to control ER stress responses in mammalian cells. The finding that exogenous XBP1 can repress the splicing of endogenous XBP1 without repressing PERK is noteworthy and suggests ways that in future, manipulation of individual branches of the UPR pathway could be used to target protein misfolding in disease, or to harness the ER more efficiently for the production of recombinant proteins. The paper is clearly written and the interpretation of the results is sound, although there are some aspects of the work that should be followed up.

In Figure 1 (lane 4), there appear to be weak protein bands induced at 75 kD and 100 kD that react with the XBP1 antisera. Since these bands do not appear in the non-induced lysates, they may be specific. It would be interesting, therefore, to determine XBP1 expression and antibody reactivity in CHO-S XB cells induced with doxycycline and then treated +/- ER stress.

The authors state that “as the mechanism for PERK and Ire1 activation requires BiP dissociation it seems unlikely that the absence of Ire1 activation is due to increased levels of BiP.” This assertion could be directly tested by examining what happens to BiP mRNA and protein levels in the overexpressing cells versus control cells (+/- ER stress).

In Figure 4, the experiments have been performed only once and the results, whilst clear, cannot be analysed statistically and are therefore somewhat preliminary. I would like to see the experiments in Figure 4 repeated, including a loading/blotting control for Figure 4C.

In Figure 4A, the flow cytometry data suggesting that ER expansion has occurred in the CHOS-XB cells is indirect, as the intracellular distribution of the dye may be influenced by stress responses. Taking this work forward in the future, it will be important to quantitate ER expansion directly e.g. in an adherent cell line, where ER shape and size can be assessed by confocal or electron microscopy.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 Jun 2017

Neil Bulleid, University of Glasgow, UK

We thank the referee for insightful comments. We are currently carrying out further experimentation to address the concerns.

Competing Interests: No competing interests were disclosed.

Author Response 28 Sep 2017

Neil Bulleid, University of Glasgow, UK

We thank the reviewer for his comments. We have now submitted a revised version of the paper and address the reviewer's comments below.

1. The weak protein bands seen in figure 1 that have a slower mobility than XBP1 are indeed only present in the induced sample and are identified with the antibody as containing XBP1. It has been shown previously that XBP1 can be modified by SUMOylation so we presume that these additional protein bands are due to this modification. We include a sentence in the results to suggest this possibility.
2. The differential effect of exogenously expressed XBP1 has now been tested by determining the effect of a range of tunicamycin concentrations on XBP1 splicing or PERK activation. This additional experiment indicates that Ire1 is activated and as sensitive to UPR inducers in the presence of exogenous XBP1 as in its absence, but its splicing activity is dramatically repressed. This result does not address the consequence of BiP dissociation from Ire1 or PERK directly but it clarifies that there is a suppression of activity rather than activation.
3. We have now carried out a repeat of Figure 4C with a loading control. (see ORF (DOI: 10.17605/OSF.IO/BGCDE;))

4. We note the suggestion by the reviewer.

Competing Interests: No competing interests were disclosed.

Referee Report 06 June 2017

doi:[10.21956/wellcomeopenres.12708.r23240](https://doi.org/10.21956/wellcomeopenres.12708.r23240)



Stefan J. Marciniak 

Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK

Protein misfolding in the endoplasmic reticulum (ER), ER stress, activates an unfolded protein response (UPR)¹. This UPR defends the cell by attenuating protein synthesis, enhancing ER chaperone levels and activity, and increasing ER-associated protein degradation. Three signalling pathways make up the UPR, each initiated by a separate ER stress sensor, IRE1 α , PERK, and ATF6. These differ in their downstream signalling and in their kinetics of activation and deactivation^{2,3}. Activated IRE1 α initiates the splicing of the mRNA encoding XBP1⁴⁻⁶. Unspliced XBP1 mRNA encodes an inactive protein, XBP1u, while spliced XBP1 mRNA encodes an active transcription factor, XBP1s. XBP1s activates genes that increase ER chaperone expression and cause expansion of the ER.

In the study of Chalmers *et al*, cells were generated that express active XBP1s protein in response to treatment with doxycycline. This caused expansion of the ER, consistent with induction of the known gene expression programme of XBP1s. When forced to express exogenous XBP1s, these cells also showed reduced splicing of endogenous XBP1u when treated with tunicamycin, an inhibitor of glycosylation commonly used to induce ER stress. In contrast, activation of PERK in response to ER stress-inducing agents (DTT, tunicamycin, or thapsigargin) appeared to remain unaffected.

This is a well-written paper. The proposed direct feedback mechanism linking XBP1s to inhibition of XBP1u splicing would be an important finding if it could be demonstrated definitively; however, the current version of the paper leaves a second (less interesting) mechanism still to be excluded.

Main concern

The authors suggest that XBP1s selectively blocks splicing of endogenous XBP1u, and that this does not involve a simple generalised resistance of the ER to stress. The authors acknowledge the second possibility in their discussion, but dismiss it because (i) PERK activation persists following induction of XBP1s and (ii) PERK and IRE1 α are known to share a similar mechanism of activation⁷. However, differences in the sensitivity of IRE1 α and PERK to ER stress could account for their observations. If PERK were to be more sensitive than IRE1 α to ER stress, then induction of BiP (or any process downstream of XBP1s that ameliorates ER stress) could block activation of IRE1 α while leaving PERK apparently unaffected. This would occur if the concentration of stressor used were to be above the threshold required to activate PERK but below that required to activate IRE1 α . To address this, ranges of DTT, tunicamycin, and thapsigargin concentrations could be tested for their effects on IRE1 α and PERK, in the presence and absence of overexpressed XBP1s. If a selective negative feedback mechanism exists linking XBP1s to the inhibition of XBP1u splicing, then the EC50s of these agents will increase only

for the activation of IRE1 α .

Minor concerns

1. It is unclear why the phosphorylation of IRE1 α has not been measured. This would help determine at what stage XBP1s antagonizes IRE1 α signaling.
2. The kinetics of activation and deactivation of IRE1 α and PERK differ significantly³. Have these been examined in this system?
3. Does expression of XBP1s inhibit the activity of an IRE1 α cytosolic domain activated independently of ER stress, e.g. the IRE1 constructs described in refs^{3,8}?

References

1. Ron D, Walter P: Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol.* 2007; **8** (7): 519-29 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Walter F, Schmid J, Düssmann H, Concannon CG, Prehn JH: Imaging of single cell responses to ER stress indicates that the relative dynamics of IRE1/XBP1 and PERK/ATF4 signalling rather than a switch between signalling branches determine cell survival. *Cell Death Differ.* 2015; **22** (9): 1502-16 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, Shokat KM, Lavail MM, Walter P: IRE1 signaling affects cell fate during the unfolded protein response. *Science.* 2007; **318** (5852): 944-9 [PubMed Abstract](#) | [Publisher Full Text](#)
4. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K: XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell.* 2001; **107** (7): 881-91 [PubMed Abstract](#)
5. Calton M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG, Ron D: IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature.* 2002; **415** (6867): 92-6 [PubMed Abstract](#) | [Publisher Full Text](#)
6. Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K, Kaufman RJ: IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.* 2002; **16** (4): 452-66 [PubMed Abstract](#) | [Publisher Full Text](#)
7. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D: Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol.* 2000; **2** (6): 326-32 [PubMed Abstract](#) | [Publisher Full Text](#)
8. Han D, Upton JP, Hagen A, Callahan J, Oakes SA, Papa FR: A kinase inhibitor activates the IRE1 α RNase to confer cytoprotection against ER stress. *Biochem Biophys Res Commun.* 2008; **365** (4): 777-83 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Sep 2017

Neil Bulleid, University of Glasgow, UK

The main concern of this reviewer was that the results could be explained if the sensitivity of Ire1 or PERK to UPR induction was different so that, at the concentrations we are using, there could be a differential effect. We carried out the experiment suggested by the reviewer, i.e. a titration of the response to tunicamycin in the absence or presence of exogenous XBP1. We include the result as a new figure in our revised version (Figure 5). Interestingly the result demonstrates that the exogenous XBP1 suppresses Ire1 splicing activity but does not alter its sensitivity towards the inducer. The conclusion is that Ire1 can still be activated but its activity is in some way inhibited by the presence of exogenous XBP1. No effect was seen for PERK which showed the same sensitivity to the UPR inducer in the presence or absence of exogenous XBP1.

The minor concerns of the reviewer can be addressed as follows:

1. We have tried several commercial antibodies to IRE1 α but none recognise the hamster protein. We can indirectly conclude that Ire1 α is activated from the splicing assays carried out but cannot assay its phosphorylation status.
2. We have not carried out an analysis of the kinetics of activation or inactivation. As our comparison is between the absence of presence of exogenous XBP1, we focused on the difference between the splicing activity towards endogenous XBP1 in this paper.
3. We have not carried out the experiments with the cytosolic domains of Ire1 α suggested by the reviewer. We do know that in the absence of an exogenous agent to activate the UPR we still see a suppression of XBP1 splicing in the presence of exogenous XBP1. This is likely due to a low level of ER stress that occurs even in the absence of external agents.

Competing Interests: No competing interests were disclosed.