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Signal peptide peptidase functions in ERAD to cleave the unfolded protein response regulator XBP1u

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Editor: Hartmut Vodermaier

1st Editorial Decision 05 March 2014

Thank you again for submitting your manuscript on SPP-Derlin-TRC8 in XBP1u proteolysis for our consideration. It has now been reviewed by three expert referees, whose comments are copied below. As you will see, at least two of the reviewers consider these findings of interest and potential importance. However, all referees remain to varying degrees unconvinced that your present data provide sufficiently compelling evidence for the proposed key role of SPP-Derlin-TRC8 in XBP1u degradation, nor for the functional significance of this process. In our view, these well-taken concerns at present preclude publication in The EMBO Journal; but in light of the principle interest of this work, we would nevertheless be inclined to give you an opportunity to improve and clarify these aspects by way of a revised version of this manuscript.

Since conclusively addressing these key issues may require substantial further efforts, and it is our policy to allow only a single round of major revision, I would in this case appreciate hearing back from you within the coming weeks how you could envision responding to these concerns, in order to be able to discuss the requirements for a successful revision of this work.

When preparing your letter of response to the referees' comments, please make sure to diligently respond to all the points raised at this stage, and also remember that this will form part of the Review Process File available online to the community in case of publication. We generally allow three months as standard revision time (limited extensions can be negotiated), and it is our policy that competing manuscripts published during this period will have no negative impact on our final

assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider this work for The EMBO Journal - I look forward to hearing from you and to eventually receiving your revision.

REFEREE COMMENTS

Referee #1:

This manuscript proposes a novel and exciting mechanism by which the intramembrane protease SPP catalyzes the cleavage and degradation of the unusual membrane protein XBP1u, which is involved in the unfolded protein response. The authors build on previous work by different labs and demonstrate that SPP does not only interact with TRC8 (as previously known), but also with Derlin1. They propose that this (at least) ternary complex has a substrate receptor function (Derlin1), a catalytic part (SPP) and a part for further degradation (TRC8). SPP is generally assumed to be preceded by a shedding event trimming the long extracellular domains. Here, the authors propose a clever model, according to which Derlin1 may bind the long ectodomain and thus make it appear short, thereby allowing SPP cleavage to occur. The manuscript is timely, novel, well written and has implications for a number of different fields, including basic cell biology, regulated intramembrane proteolysis and the UPR field.
My major criticism is that the manuscript remains somewhat preliminary for two reasons: First, I am not yet fully convinced of the central role of SPP in XBP1u cleavage, degradation and function. Second, the functional relevance of XBP1u cleavage/degradation by the SPP complex remains unclear.

The following points should be addressed:

Major points:
1. Epoxomicin stabilizes XBP1u in Fig. 1F, whereas the SPP inhibitors ZLL ketone and L685,458 only do partly so. If there really was a pathway, in which SPP acts upstream and makes the cut in the transmembrane domain of XBPu1, while the proteasome acts downstream and mediates degradation, then I would expect that blocking either step (SPP or proteasome) should have the identical effect. But here, proteasome inhibition has a stronger effect than SPP inhibition, making it likely that other proteases can cleave XBP1u as well. If the authors do not have strong evidence against my point, this issue should be discussed. Moreover, how can the authors exclude the possibility that the 'weak' transmembrane domain of XBP1u may integrate into the membrane both in a type I and a type II orientation? One orientation would then be cleaved by SPP, the other one by gamma-secretase or a rhomboid. Such a dual orientation - although rarely seen in proteins - may also explain the partial glycosylation.
2. The authors demonstrate that SPP overexpression enhances cleavage. But this only shows that SPP CAN cleave XBP1u, but - like for many other proteases - this does not yet prove the cleavage under physiological conditions. Moreover, although both pharmacological inhibitors have previously been shown to block SPP, such drugs are rarely specific. In fact, L685 has initially been identified as a potent inhibitor of gamma-secretase. Interestingly, L685 induces a much stronger accumulation of XBP1u than the more specific SPP inhibitor ZLL ketone. Similar to point 1 this may indicate a role of gamma-secretase in an alternatively oriented XBP1u. To resolve this issue the authors need to knock-down SPP and presenilin (or use the corresponding knock-out cells) and demonstrate the selective role of SPP and not of presenilin/gamma-secretase.
3. If SPP was the only protease cleaving XBP1u, I would expect that cleavage of the truncated XBP1u Delta should be abolished by the catalytically inactive SPP D265A mutant, resulting in a half-life similar to wt XBP1u. However, degradation of this mutant XBP1u is still strongly enhanced compared to wt XBP1u, strongly arguing for additional proteases cleaving this XBP1u.
4. The proteolytic processing of XBP1u is only partially characterized in the manuscript. While suitable antibodies may not be available detecting the N- and C-terminal fragments, the authors should simply generate a double-tagged XBP1u protein and show the N- and C-terminal cleavage fragments in the presence or absence of the endogenous SPP.
5. In the current version of the manuscript it is tempting to speculate that Derlin1-XBP1u interaction obviates the need for a prior shedding event. However, evidence for this mechanism is not yet very

strong. The model would predict that many other ectodomains should also slow down SPP cleavage of XBP1u. This needs to be tested, either with a chimeric ectodomain or potentially even with the the ectodomain of the protein described in the Voss et al publication (cited by the authors). While that protein does not require shedding when being cleaved by SPPL3, this protein's ectodomain may well block cleavage by SPP if fused to the XBPu1 transmembrane domain.
6. The physiological relevance of the XBP1u cleavage remains enigmatic. The authors make a prediction based on their model (page 16 end of first paragraph , ER-stressed cells) which can easily be tested.

Referee #2:

SUMMARY
In this manuscript, the authors explore a putative role for the intramembrane protease signal peptide peptidase (SPP) in ER-associated degradation (ERAD). In doing so, they identify and characterize and interactions of SPP with Derlin1, an established ERAD component and putative rhomboid-like protease, and Trc8, an ER-resident ubiquitin ligase implicated in previously in US2-induced degradation of MHC Class I. They describe a regulatory protein of the Unfolded Protein Response (UPR), XBP1u (unspliced) as a substrate of SPP. They demonstrate that XBP1u adopts a type II orientation by virtue of a transmembrane domain that has so far been uncharacterized. Moreover, the authors suggest that Derlin1 acts in the context of a receptor for XBP1u in order for SPP to access it. XBP1u does not seem to lose its ectodomain due to an interaction with Derlin1. The authors also provide evidence that the XBP1u form attenuates XBP1s action (and hence UPR activation) by targeting it for degradation. Collectively, the authors propose an SPP regulated mechanism of UPR tuning through degradation of XBP1s via XBP1u.

The findings presented by Chen et al. reveal several novel aspects regarding the relationship between ERAD and UPR regulation. They demonstrate the presence of Derlin1 in a 500kD complex with SPP and Trc8 that also binds XBP1u. They convincingly show that XBP1u can adopt the conformation of a type II membrane protein, for which the hydrophobic sequence responsible is cleaved by SPP. A role for Derlin1-mediated recognition of the XBP1u ectodomain is also clearly demonstrated. Overall the manuscript was well written and the data clearly presented. The authors have cited appropriate references have provided a sufficient number of supplemental figures to complement the primary data. Although of very good quality overall, there are several issues with both text and the data that should be addressed. These are briefly mentioned below.

QUESTIONS

Question 1. In the Lee (2010) study cited, an interaction between SPP and Derlin1 was not observed. Can the authors offer an explanation as to this difference with Figure 1A? Detergent solubisation conditions perhaps? If so, what might this say about the stability/assembly of such a complex?

Question 2. Of note is that XBP1u only seems to be partially in a membrane-integrated state (Figure 2C, 2D). The authors postulate that inefficient glycosylation is the reason why only a fraction of XBP1u-N-FLAG has its glycan acceptor site modified (Figure 2D) but Figure 2E (bottom) depicts what appears to be very little loss of XBP1-C-GFP signal from the cell in question. This raises the question, what is the relative fraction of type II to membrane-associated XBP1u? Figure E2C indicates that 75% is protected but that does not seem to agree with Figure 2D (R223N). Can the authors please explain and/or speculate on the potential for multiple XBP1u orientations.

Question 3. Along similar lines, can the XBP1u subcellular localization/orientation be recapitulated with endogenous forms? What is the relative expression level of FLAG-XBP1u to endogenous? Could the elevated, exogneous expression be causing XBP1u to adopt an Type II conformation?

Question 4. In Figure 2F, can ubiquitinated XBP1u be detected or all Ub chains removed during sample processing? In general, some demonstration of Ub conjugation would be useful to support an active role for Trc8 in this complex.

Question 5. For the mutants tested in Figure 3, was their orientation with respect to ER membrane determined (as in Figure 2) and did they adopt a type II orientation with the same frequency as the wild type?

Question 6. The 25kD fragment detected in Figure 2F is very weak and would not appear to correlate with the levels observed in Figure 4B. Can the authors comment on the relative levels of this fragment appear so different?

Question 7. In Figure 5B, no difference in the amount XBP1u co-IP with SPP WT or DA is observed. If XBP1u is unable to be processed by SPP-DA, I would have expected that more might be observed. Can the authors shed some light on why no change was observed?

Question 8. In Figure 7A and 7B, several prominent bands listed as "unidentifiable co-immunoprecipitated proteins" are listed. There is a striking difference between XBP1u and XBP1uΔ with respect to these bands. For instance, the higher band observed in SPP-D286A is missing as is the lowest band of DerlinG189V. It would appear that these bands represent interesting interactors or modified XBP1u forms. Could the authors address what they might represent or perhaps determine at least whether they represent XBP1u (modified) or a completely different protein.

Question 9. Is the stabilizing effect of the ectodomain specific for the sequence or merely for the presence of additional sequence following the TM domain? Specifically, would the ectodomain be sufficient for Derlin1 interaction if a chimera is made with a different type II protein?

Question 10. Does the Derlin1-G180V mutant still assemble with SPP-Trc8 in the 500kD complex?

Question 11. Is Derlin1 still present along with SPP-Trc8 in complexes when XBP1uΔ is expressed? Specifically, the temporal aspects of these interactions are not entirely clear. Is Derlin1 is brought to the complex by XBP1u ectodomain or is it poised within the 500kD complex, waiting for XBP1u for come along?

MINOR COMMENTS
Comment 1. The resolution of images in Figure E2G makes interpretation a bit difficult.

Comment 2. Pg. 14, first sentence. Fig 2D should be Fig 2F

Comment 3. Pg 16, the sentence beginning with "Although different half-lives... " is grammatically confusing. Perhaps it should be rewritten.

Comment 4. Pg. 17. 2nd paragraph, 2nd sentence.. "...which extends..", I think should be "...what extent...".

Referee #3:

Contrary to the current consensus in the field that XBP1u is a peripheral membrane protein in the ER (located entirely on the cytosolic side), the authors claim that XBP1u is synthesized as a type II transmembrane protein in the ER and degraded by a 500-kDa ERAD complex consisting of SPP, Derlin-1 and TRC8. However, if intramembrane cleavage of XBP1u by SPP triggers subsequent proteosomal degradation, the authors must observe both full length XBP1u and cleaved form of XBP1u in pulse chase experiments. This reviewer cannot believe the authors' story until the authors provide convincing data for the two-step cleavage of XBP1u (first by SPP and second by proteasome) by pulse chase experiments. N-terminal fragment of XBP1u observed was too faint in cycloheximde chase experiment (Fig. 2F) to support the authors' claim.

If the authors insist that "Despite that, upon inhibiting the proteasome we observed mainly accumulation of the full-length XBP1u and only traces of the SPP-generated cleavage fragment (Fig 2D and 4C). Since epoxomicin is specific for the proteasome and does not inhibit SPP (Bland et al, 2003), the stabilization effect on the full-length form indicates that under our experimental

conditions there is a so far unrecognized functional coupling between SPP and the proteasome, which is known to associate with the ER-membrane (Palmer et al., 1996)" (p.13, line 2 from the bottom - p.14, line 6), the authors should uncover the coupling mechanism for this manuscript to be published in EMBO J. This is a critical point.

Identification of the 500-kDa SPP-Derlin1-TRC8 complex was interesting to this reviewer, however, the XBP1u part probably represents an artifact resulting from overexpression of XBP1u, because there are a lot of discrepancy in the data.

Discrepancy
1) a) Immunofluorescence; XBP1u-N-GFP is localized only in the ER in Fig. 2B. However, bright fluorescence from XBP1u-N-GFP and XBP1u-C-GFP was seen in the nucleus (Fig. 2E and Fig. E2A and E2B).
1) b) Fig. 2F; XBP1u exists as full-length (ER) form in epoxomicin-treated cells, whereas bright fluorescence was observed in the nucleus of epoxomicin-treated cells expressing XBP1u-GFP in Fig. 4B.
1) c) Only half of XBP1u remained associated with ER membrane after sodium carbonate wash in Fig. 2C. Only small fraction of FLAG-XBP1u was glycosylated (Fig. 2D).
Despite these facts, degradation of XBP1u was blocked almost completely by co-expression with SPP mutant D265A in Fig. 5A, which seems too effective than expected to this reviewer. The authors must show how much of XBP1u stays in the ER as a type II transmembrane protein quantitatively.

2) Fig. 6; why did cleavage-deficient XBP1u mutant mt2 reduce expression of UPR reporter? If the authors' claim is correct, XBP1u mutant mt2 remains associated with the ER, and therefore is unable to down regulate XBP1s. The data in Fig. E4 contradict the authors' claim.

Specific points
1) Fig. 2G; show the band of XBP1s in western and show the bands of cDNA corresponding to XBP1s and XBP1u mRNA by RT-PCR to say that epoxomicin treatment reduced XBP1u levels probably by IRE1-mediated reduction of the XBP1 pre-mRNA, which upon accumulation of unfolded proteins activates the canonical UPR signaling.
2) Fig. 3A; show the localization and topology of the two mutants and examine whether the two mutants are cleaved or not cleaved by SPP as in Fig. 4C.

1st Revision - authors' response 16 June 2014

We are pleased that two referees were positive about our paper, and thank all three referees for their helpful comments. We now provide several new experiments addressing the previous shortcomings of our manuscript and further supporting our claims:

- We now apply an additional approach to confirm topology of XBP1u as a type II membrane protein. Importantly, this new strategy allowed us to demonstrate that XBP1u persists in this membrane-spanning form when trapped by the catalytic SPP^{D265A} mutant. Over all, in the revised manuscript, we provide evidence by four independent methods that the predominant fraction of XBP1u is an ER membrane protein with a type II topology.
- We show that the g-secretase-specific inhibitor DAPT, even at very high dose, does not interfere with XBP1u turnover, providing strong evidence that residual degradation in presence of SPP inhibitors is not caused by g-secretase-catalyzed cleavage.
- We detect the C-terminal fragment generated by SPP-catalyzed XBP1u cleavage and show that, like the N-terminal fragment, also this luminal portion is efficiently degraded by the ERAD machinery.
- By western analysis of immunoisolated XBP1u we show that it is ubiquitinated; moreover, we provide evidence that this step is mediated by the E3 ligase TRC8.
- Domain swap and pulse-chase experiments provide further evidence that, in addition to determinants in the XBP1u transmembrane domain, also the C-terminal portion contributes to recognition by the SPP-Derlin1-TRC8 complex. Intriguingly, this tail was sufficient to

drive degradation of a stable type II membrane protein. This highlights the role of Derlin1 in recruiting XBP1u to SPP for subsequent proteolytic degradation.

Moreover, we provided additional controls as requested, and sharpen our claims concerning the influence of XBP1u in UPR tuning. In the course of the revision, we have changed the order of the figures now showing first the data on the UPR tuning (Fig 5. Inhibition of UPR by XBP1u is independent of SPP-catalyzed cleavage) before focusing on the molecular mechanism of how the SPP ERAD complex functionally interacts with XBP1u (Fig 6. XBP1u is specifically recognized by SPP-Derlin1-TRC8 complex). Last, we have changed our title, which now better summarizes our main scientific claims. Over all, we are convinced that we now can satisfy all critical issues that have been raised and this indeed strengthens our conclusions dramatically. Below we provide a point-by-point response to the referees' comments (*answers in italics*).

Referee #1:

This reviewer asked us to address the following points:

1. Epoxomicin stabilizes XBP1u in Fig. 1F, whereas the SPP inhibitors ZLL ketone and L685,458 only do partly so. If there really was a pathway, in which SPP acts upstream and makes the cut in the transmembrane domain of XBPu1, while the proteasome acts downstream and mediates degradation, then I would expect that blocking either step (SPP or proteasome) should have the identical effect. But here, proteasome inhibition has a stronger effect than SPP inhibition, making it likely that other proteases can cleave XBP1u as well. If the authors do not have strong evidence against my point, this issue should be discussed. Moreover, how can the authors exclude the possibility that the 'weak' transmembrane domain of XBP1u may integrate into the membrane both in a type I and a type II orientation? One orientation would then be cleaved by SPP, the other one by gamma-secretase or a rhomboid. Such a dual orientation - although rarely seen in proteins - may also explain the partial glycosylation.

This is a fair point. Although we think the more pronounced effect of epoxomicin is caused by a better pharmacokinetic of this compound, in the revised version of the manuscript we discuss (on page 18) that XBP1u may have alternative fates and that redundant degradation routes may exist. However, in absence of an N-terminal signal sequence, insertion of XBP1u as a type I membrane protein is rather unlikely (the bulky bZIP domain can not flip and pass the narrow Sec61 translocon), and therefore in the manuscript we do not discuss such a hypothetical inverted topology.

2. The authors demonstrate that SPP overexpression enhances cleavage. But this only shows that SPP CAN cleave XBP1u, but - like for many other proteases - this does not yet prove the cleavage under physiological conditions. Moreover, although both pharmacological inhibitors have previously been shown to block SPP, such drugs are rarely specific. In fact, L685 has initially been identified as a potent inhibitor of gamma-secretase. Interestingly, L685 induces a much stronger accumulation of XBP1u than the more specific SPP inhibitor ZLL ketone. Similar to point 1 this may indicate a role of gamma-secretase in an alternatively oriented XBP1u. To resolve this issue the authors need to knock-down SPP and presenilin (or use the corresponding knock-out cells) and demonstrate the selective role of SPP and not of presenilin/gamma-secretase.

Despite our long-standing effort to knockdown SPP – by transient siRNA transfection and generation of stable shRNA-expressing cells – unfortunately, we have not managed to reduce the protease level sufficient enough to see robust effects (also on all our previously identified control substrates such as the HCV core protein). Hence, we use two chemically distinct SPP inhibitors to interfere with endogenous SPP activity and carefully restrict our claim (on page 10) "that this result strongly suggests that XBP1u is degraded in an SPP-dependent manner". Of note, we previously showed that due to its extreme hydrophobicity (Z-LL)$_2$-ketone shows only modest inhibition of SPP in cells and therefore also used the more potent drug L-685,458 (Weihofen et al., 2003), which initially has been developed as presenilin/ g-secretase inhibitor. To clarify the referee's concern that g-secretase may contribute to XBP1u turnover (despite the common believe that it only acts on type I membrane proteins), we now show that a high dose of g-secretase inhibitor (DAPT, 50 µM), which does not cross-react with SPP

(Weihofen et al., 2003), has no influence on XBP1u turnover (Fig 2F).

3. If SPP was the only protease cleaving XBP1u, I would expect that cleavage of the truncated XBP1u Delta should be abolished by the catalytically inactive SPP D265A mutant, resulting in a half-life similar to wt XBP1u. However, degradation of this mutant XBP1u is still strongly enhanced compared to wt XBP1u, strongly arguing for additional proteases cleaving this XBP1u.

*The pulse chase assay clearly shows that XBP1uD functionally interacts with SPP, Nevertheless, we agree that the residual decay indicates that, upon blocking its physiological degradation route by SPP^{D265A}, alternative pathways take over. This redundancy is very characteristic for protein degradation at the ER, and has led to the suggestion of an adaptive organization of the ERAD pathway (Christianson et al., 2011). However, we cannot make any prediction whether an alternative intramembrane protease takes over or instead XBP1uD is degraded by one of the canonical ERAD dislocation routes. Accordingly, we have rephrased the relevant sentence (on page 18), which now reads: "*The relatively high turnover rate compared to the full-length substrate indicates that, upon blocking the SPP-dependent pathway, other ERAD branches are capable of degrading XBP1u *(Christianson et al., 2011)."*

4. The proteolytic processing of XBP1u is only partially characterized in the manuscript. While suitable antibodies may not be available detecting the N- and C-terminal fragments, the authors should simply generate a double-tagged XBP1u protein and show the N- and C-terminal cleavage fragments in the presence or absence of the endogenous SPP.

We have addressed this point and now show the fate of the C-terminal fragment (Fig E4D). Interestingly this fragment is efficiently degraded by the proteasome in the manner of a soluble ERAD substrate.

5. In the current version of the manuscript it is tempting to speculate that Derlin1-XBP1u interaction obviates the need for a prior shedding event. However, evidence for this mechanism is not yet very strong. The model would predict that many other ectodomains should also slow down SPP cleavage of XBP1u. This needs to be tested, either with a chimeric ectodomain or potentially even with the the ectodomain of the protein described in the Voss et al publication (cited by the authors). While that protein does not require shedding when being cleaved by SPPL3, this protein's ectodomain may well block cleavage by SPP if fused to the XBPu1 transmembrane domain.

We followed on this interesting idea and performed domain swap experiments with the type II membrane protein invariant chain (Fig 8A-C and E8A-B). Interestingly, this new data shows that the C-terminal tail of XBP1u alone is sufficient to target a type II membrane protein to the SPP-dependent ERAD pathway. By showing that removal of the tail obviates the need for Derlin1 in XBP1u degradation (Fig 7B and E8C) and that, vice versa, the tail fused to a substrate of the endosomal SPPL2a protease targets it to SPP (Fig 8B), we now provide compelling evidence for our claims.

6. The physiological relevance of the XBP1u cleavage remains enigmatic. The authors make a prediction based on their model (page 16 end of first paragraph, ER-stressed cells) which can easily be tested.

Since inhibition of SPP is expected to cause accumulation of other substrates and likely off-target effects are observed, we have chosen to block XBP1u turnover by specifc point mutations in the XBP1u transmembrane domain. This revealed that the membrane-anchored form triggers degradation of ectopically expressed XBP1s (Fig 5A and E5) and interferes with the endogenous UPR signaling machinery (Fig 5B). Since previous reports already demonstrated a physiological role of XBP1u in regulating UPR transcription factors (Yoshida, 2006; Tirosh, 2006; Yoshida, 2009), we restrict our claims here to a new facet of XBP1u abundance control. Accordingly, we have removed any unnecessary speculation in the results section and sharpened our claims (on page 16) "that membrane-anchored XBP1u is a physiological inhibitor of the UPR." Moreover we conclude (on page 23) that "SPP-mediated release of XBP1u from the membrane triggers its proteasomal degradation and thereby provides a mechanism to regulate XBP1u abundance", which "indirectly modulates the inhibitory role of XBP1u during UPR signaling." Moreover, we have

changed the order of Figures 5 and 6, and believe that this makes the paper now easier to read.

Referee #2:

This reviewer asked us to address the following points:

Question 1. In the Lee (2010) study cited, an interaction between SPP and Derlin1 was not observed. Can the authors offer an explanation as to this difference with Figure 1A? Detergent solubisation conditions perhaps? If so, what might this say about the stability/assembly of such a complex?

The analysis by Lee et al. (2010) was performed in HeLa cells expressing the human cytomegalovirus protein US2, which as a part of an immune escape mechanism drives proteolytic downregulation of MHC-I molecules, likely affecting the entire ERAD network. Since we observed robust interaction between SPP and Derlin1 using two chemically distinct detergents, including digitonin as used by Lee et al, we reason that, in uninfected cells, the complex is quite stable and the interaction is physiologically relevant.

Question 2. Of note is that XBP1u only seems to be partially in a membrane-integrated state (Figure 2C, 2D). The authors postulate that inefficient glycosylation is the reason why only a fraction of XBP1u-N-FLAG has its glycan acceptor site modified (Figure 2D) but Figure 2E (bottom) depicts what appears to be very little loss of XBP1-C-GFP signal from the cell in question. This raises the question, what is the relative fraction of type II to membrane-associated XBP1u? Figure E2C indicates that 75% is protected but that does not seem to agree with Figure 2D (R223N). Can the authors please explain and/or speculate on the potential for multiple XBP1u orientations.

In order to further support our claim that the predominant pool of XBP1u is a type II membrane protein, we studied by immunofluorescence analysis accessibilityof the C-terminal HA-tagged XBP1u upon selective permeabilization of the plasma membrane (Fig E2D). As stated on page 9 we assume that the apparent discrepancy in the glycosylation efficiency in Fig 2D and the protected fraction in Fig E2C is due to a relatively inefficient glycosylation site. Of note, it is well documented that oligosaccharyltransferase (OST) not always quantitatively modifies newly synthesized proteins, which for our artificially generated XBP1u mutants leads only to a moderate glycosylation. Such partial glycosylation is also observed for physiological OST substrates such as pancreatic ribonuclease, known as RNAse A for the unmodified form and RNAse B for the glycosylated species (Rainer, Chem. Rev. 1998).

Over all, we now provide evidence for this membrane-spanning topology by using four independent assays, namely 1.) analysis of a glycosylation mutant (Fig 2D),
2.) fluorescent protease protection assays (Fig 2E), 3.) selective permeabilization and immunofluorescence analysis (Fig E2D), and 4.) in vitro translocation and protease protection (Fig E2E). This includes quantitative data of the fluorescent protease protection assay demonstrating that the C-terminus of XBP1u is protected in the range of a luminal control protein (Fig E2C). Of note, all previous reports on that issue did not experimentally investigate XBP1u topology (Yoshida et al., 2006; Yanagitani et al., 2009). Despite our strong evidence of a type II topology of XBP1u, we follow on the reviewer's suggestion and indicate (on page 9) "that a fraction of XBP1u may be peripheral attached as stalled nascent chains and translocation intermediates".

Question 3. Along similar lines, can the XBP1u subcellular localization/orientation be recapitulated with endogenous forms? What is the relative expression level of FLAG-XBP1u to endogenous? Could the elevated, exogneous expression be causing XBP1u to adopt an Type II conformation?

We show by immunofluorescence microscopy that endogenous XBP1u co-localizes with an ER maker (Fig E2I), supporting the claim of membrane attached XBP1u. Unfortunately, no antibody detecting the luminal domain of XBP1u exits, preventing us from performing a protease protection assays with endogenous protein. However, to our knowledge, there is no report that the topology of a single-spanning membrane protein is affected by the expression level. We believe that, by validating the topology of XBP1u by four independent assays (see above), we dramatically strengthen the notion that it is physiological relevant.

Question 4. In Figure 2F, can ubiquitinated XBP1u be detected or all Ub chains removed during

sample processing? In general, some demonstration of Ub conjugation would be useful to support an active role for Trc8 in this complex.

We followed on that question and provide evidence that XBP1u is polyubiquitianted (Fig E4B, E4D and E7C).

Question 5. For the mutants tested in Figure 3, was their orientation with respect to ER membrane determined (as in Figure 2) and did they adopt a type II orientation with the same frequency as the wild type?

We validated type II topology of both mutants by a fluorescent protease protection assays (Fig E3A-B).

Question 6. The 25kD fragment detected in Figure 2F is very weak and would not appear to correlate with the levels observed in Figure 4B. Can the authors comment on the relative levels of this fragment appear so different?

Experimental hurdles such as post-lysis degradation or inefficient transfer by western blotting makes detection of peptide fragments by SDS-PAGE challenging. Microscopy analysis shown in Fig 4B, however, is performed in living cells, allowing to demonstrate the real extend of peptide release.

Question 7. In Figure 5B, no difference in the amount XBP1u co-IP with SPP WT or DA is observed. If XBP1u is unable to be processed by SPP-DA, I would have expected that more might be observed. Can the authors shed some light on why no change was observed?

We acted on that concern and now show a quantification of XBP1u co-purified with SPP wt and SPP^{D265A} (Fig E6B), revealing slight differences in the trapping efficiency. Despite that, we comment on the unexpected observation that „also SPP wt co-purified XBP1u although with a slightly reduced efficiency (Fig 6B)". Of note, a recent enzymological characterization of the E. coli rhomboid intramembrane proteases GlpG has revealed a surprising low catalytic rate (Dickey et al., 2013). Hence, we discuss (on page 16) that our result suggests "that the SPP-reaction cycle is slow", which likely is a multi-step reaction with potentially other rate limiting steps than proteolysis, given that in the 500-kDa SPP complex different factors act on the substrate.

Question 8. In Figure 7A and 7B, several prominent bands listed as "unidentifiable co-immunoprecipitated proteins" are listed. There is a striking difference between XBP1u and XBP1uΔ with respect to these bands. For instance, the higher band observed in SPP-D286A is missing as is the lowest band of DerlinG189V. It would appear that these bands represent interesting interactors or modified XBP1u forms. Could the authors address what they might represent or perhaps determine at least whether they represent XBP1u (modified) or a completely different protein.

This observation is well taken, but unfortunately analysis of these factors is not straightforward, and since we think this is not central to the manuscript we will not follow on that point.

Question 9. Is the stabilizing effect of the ectodomain specific for the sequence or merely for the presence of additional sequence following the TM domain? Specifically, would the ectodomain be sufficient for Derlin1 interaction if a chimera is made with a different type II protein?

As discussed in the response to reviewer #1 (point 5), we performed domain swap experiments with the type II membrane protein invariant chain (Fig. 8A-C and E8A-B). Interestingly, these new pulse-chase experiments show that the C-terminal tail of XBP1u alone is sufficient to target a type II membrane protein to the SPP-dependent ERAD pathway.

Question 10. Does the Derlin1-G180V mutant still assemble with SPP-Trc8 in the 500kD complex?

We show by BN-PAGE analysis of immunoisolated SPP that $Derlin1^{G180V}$ assembles in the 500 kDa complex (Fig E7A).

Question 11. Is Derlin1 still present along with SPP-Trc8 in complexes when XBP1uΔ is expressed? Specifically, the temporal aspects of these interactions are not entirely clear. Is Derlin1 is brought to the complex by XBP1u ectodomain or is it poised within the 500kD complex, waiting for XBP1u for come along?

Similarly, we used BN-PAGE to resolve this issue and favor a model where Derlin1 is part of the 500-kDA complex prior to XBP1u binding (Fig E7F).

MINOR COMMENTS

Comment 1. The resolution of images in Figure E2G makes interpretation a bit difficult.

Since indirect immunofluorescence analysis of fixed cells does not allow a higher resolution (Fig E2I, previous Fig E2G), we carefully restrict our claims and just indicate that "immunofluorescence analysis of fixed Hek293T cells showed an accumulation of endogenous XBP1u in the ER upon SPP inhibition (Fig 2H and E2I), whereas upon epoxomicin treatment we observed accumulation of a fuzzy XBP1u signal adjacent to the ER (Fig E2I)".

Comment 2. Pg. 14, first sentence. Fig 2D should be Fig 2F

Comment 3. Pg 16, the sentence beginning with "Although different half-lives... " is grammatically confusing. Perhaps it should be rewritten.

Comment 4. Pg. 17. 2nd paragraph, 2nd sentence.. "...which extends..", I think should be "...what extent...".

We revised the manuscript accordingly.

Referee #3:

This reviewer brings up critical points that we discuss in the following.

Contrary to the current consensus in the field that XBP1u is a peripheral membrane protein in the ER (located entirely on the cytosolic side), the authors claim that XBP1u is synthesized as a type II transmembrane protein in the ER and degraded by a 500-kDa ERAD complex consisting of SPP, Derlin-1 and TRC8. However, if intramembrane cleavage of XBP1u by SPP triggers subsequent proteosomal degradation, the authors must observe both full length XBP1u and cleaved form of XBP1u in pulse chase experiments. This reviewer cannot believe the authors' story until the authors provide convincing data for the two-step cleavage of XBP1u (first by SPP and second by proteasome) by pulse chase experiments. N-terminal fragment of XBP1u observed was too faint in cycloheximde chase experiment (Fig. 2F) to support the authors' claim.

The referee is right that upon inhibition of the proteasome, we do not see full conversion of XBP1u to soluble cleavage fragments (an observation that we point out on page 11 and 14). Similarly, for most substrates of classical dislocation pathways, proteasome inhibitor treatment leads to substrate accumulation in the ER membrane fraction (and only to partial dislocation into the cytosol). Because of this feedback inhibition between the proteasome and the SPP-dependent ERAD machinery, we cannot work out a substrate product relationship.

If the authors insist that "Despite that, upon inhibiting the proteasome we observed mainly accumulation of the full-length XBP1u and only traces of the SPP-generated cleavage fragment (Fig 2D and 4C). Since epoxomicin is specific for the proteasome and does not inhibit SPP (Bland et al, 2003), the stabilization effect on the full-length form indicates that under our experimental conditions there is a so far unrecognized functional coupling between SPP and the proteasome, which is known to associate with the ER-membrane (Palmer et al., 1996)" (p.13, line 2 from the bottom - p.14, line 6), the authors should uncover the coupling mechanism for this manuscript to be published in EMBO J. This is a critical point.

The referee is right that it would be nice to understand how inhibiting the proteasome interplays with earlier steps, also blocking ERAD on the level of the ER membrane. However, given the extreme experimental challenges deciphering this problem, this analysis is beyond the scope of this manuscript.

Identification of the 500-kDa SPP-Derlin1-TRC8 complex was interesting to this reviewer, however, the XBP1u part probably represents an artifact resulting from overexpression of XBP1u, because there are a lot of discrepancy in the data.

Discrepancy

1) a) Immunofluorescence; XBP1u-N-GFP is localized only in the ER in Fig. 2B. However, bright fluorescence from XBP1u-N-GFP and XBP1u-C-GFP was seen in the nucleus (Fig. 2E and Fig. E2A and E2B).

We have revisited the original data and selected a more representative picture that also shows XBP1-N-GFP in the nucleus, although at a very low level (Fig 2B). Hence we have changed our wording, now stating (on page 8) that "GFP-tagged XBP1u co-localized with the ER maker RFP-KDEL and only weak cytosolic or nuclear signals were detected".

1) b) Fig. 2F; XBP1u exists as full-length (ER) form in epoxomicin-treated cells, whereas bright fluorescence was observed in the nucleus of epoxomicin-treated cells expressing XBP1u-GFP in Fig. 4B.

As discussed above, inhibition of the proteasome leads, besides of accumulation of the SPP-released N-terminal fragment, also to stabilization of the full-length XBP1u. Due to experimental differences, western analysis of a cycloheximide chase experiment (Fig 2F) and fluorescence microscopy analysis under steady state conditions (Fig 4B) reveal these two populations to a different extend. We explicitly indicate this discrepancy. In the results section we now conclude that our results in living cells more clearly show that degradation of XBP1u is triggered by SPP-catalyzed release that is followed by proteasomal degradation of the N-terminal fragment.

1) c) Only half of XBP1u remained associated with ER membrane after sodium carbonate wash in Fig. 2C. Only small fraction of FLAG-XBP1u was glycosylated (Fig. 2D).

This is not a discrepancy. Sodium carbonate extraction depends on the properties of the transmembrane anchor, which because of the high content of polar residues, leads only to a partial recovery of XBP1u in the pellet fraction. To clarify this point, we have changed our wording and the relevant sentence (on page 9) now reads: "Upon extraction of this fraction with high salt or sodium carbonate, approximately half of XBP1u remained membrane-associated, which is typical for a TM anchor with modest hydrophobicity."

Moreover, as noted in the response to referee #2, the oligosaccharyltransferase (OST) does not always quantitatively modify newly synthesized proteins. Hence, we assume (on page 9) „that XBP1u^{R232N} is only partially modified because the glycosylation sequence we introduced "is relatively inefficient."Of note, partial glycosylation is also observed for physiological OST substrates such as pancreatic ribonuclease, known as RNAse A for the unmodified form and RNAse B for the glycosylated species (Rainer, Chem. Rev. 1998). Despite that, we did not intend to exclude that XBP1u has also other fates, and hence indicate (on page 9) "that a fraction of XBP1u may be peripheral attached as stalled nascent chains and translocation intermediates, as has been suggested (Yanagitani et al., 2009)."

Despite these facts, degradation of XBP1u was blocked almost completely by co-expression with SPP mutant D265A in Fig. 5A, which seems too effective than expected to this reviewer. The authors must show how much of XBP1u stays in the ER as a type II transmembrane protein quantitatively.

We show by immunofluoresnce microscopy of SPP^{D265A} expressing cells that XBP1u remains a type II membrane protein (Fig E6A).

2) Fig. 6; why did cleavage-deficient XBP1u mutant mt2 reduce expression of UPR reporter? If the authors' claim is correct, XBP1u mutant mt2 remains associated with the ER, and therefore is unable to down regulate XBP1s. The data in Fig. E4 contradict the authors' claim.

We do not suggest that SPP-catalyzed cleavage contributes to silencing of XBP1s. The relevant section on page 15 reads "...the phenocopy observed for XBP1u^{mt2} suggests that SPP-catalyzed release of XBP1u is dispensable for down regulation of XBP1s." Moreover, we conclude in the discussion section (on page 23) that the „SPP-mediated release of XBP1u from the membrane triggers its proteasomal degradation and thereby provides a mechanism to regulate XBP1u abundance. This then indirectly modulates the inhibitory role of XBP1u during UPR signaling.."

Specific points

1) Fig. 2G; show the band of XBP1s in western and show the bands of cDNA corresponding to XBP1s and XBP1u mRNA by RT-PCR to say that epoxomicin treatment reduced XBP1u levels probably by IRE1-mediated reduction of the XBP1 pre-mRNA, which upon accumulation of unfolded proteins activates the canonical UPR signaling.

We show by an RT-PCR assay that under these conditions UPR is induced (Fig E2G).

2) Fig. 3A; show the localization and topology of the two mutants and examine whether the two mutants are cleaved or not cleaved by SPP as in Fig. 4C.

We validated the type II topology of both mutants by a fluorescent protease protection assays (Fig E3A-B). Since both mutants are not degraded the SPP-dependent ERAD pathway (Fig 3A), we conclude that they are not cleaved.

2nd Editorial Decision 01 July 2014

Thank you for submitting your revised manuscript for our consideration. It has now been reviewed once more by two of the original referees, whose comments are copied below. While both reviewers consider the paper generally improved and most points satisfactorily addressed, they still have a couple of specific points that would require further modification of the manuscript - in particular the major concern of referee 1 and the related point 4 of referee 2. Since I agree with referee 1 that these issues potentially affect the main conclusions of the study, I am thus returning the study to you for one additional, final round of revision, in order to clarify these points.

I hope you will be able to make the necessary further minor revisions (which I consider justified given the importance of the topic and the potential significance of the conclusions) and resubmit a final version of the manuscript (together with a response letter) as early as possible. Should you have any further questions in this regard, please do not hesitate to get back to me.

REFEREE COMMENTS

Referee #1:

Chen and colleagues have significantly improved their manuscript and adequately addressed most of my previous points. However, I am still puzzled about the extent by which XBP1u cleavage occurs by SPP. Is cleavage by SPP only a minor fraction of total XBP1u cleavage (in which case the whole story would be of less general interest)? Or does SPP mediate a large fraction of total XBP1u cleavage, making it a finding of wider interest. Fig 2G suggests that the SPP inhibitors strongly stabilize the endogenous XBP1u, which would argue for a more central role of SPP in XBP1u cleavage. In contrast the newly added Figure E4D suggests that a stabilization (inhibition of cleavage) of this tagged XBP1u only occurs with epoxomycin (proteasome inhibitor), but not with the SPP inhibitors, suggesting predominant proteasome cleavage.

The authors should address this point.

Referee #2:

SUMMARY

In this revised manuscript, the authors explore a putative role for the intramembrane protease signal peptide peptidase (SPP) in ER-associated degradation (ERAD). In doing so, they identify and characterize and interactions of SPP with Derlin1, an established ERAD component and putative rhomboid-like protease, and Trc8, an ER-resident ubiquitin ligase implicated in previously in US2-induced degradation of MHC Class I. They describe a regulatory protein of the Unfolded Protein Response (UPR), XBP1u (unspliced) as a substrate of SPP. They now include data using 4 different methods to demonstrate that XBP1u adopts a type II orientation by virtue of a previously uncharacterised transmembrane domain. The authors present data that Derlin1 acts in the context of a receptor for XBP1u in order for SPP to access it. XBP1u does not seem to lose its ectodomain due to an interaction with Derlin1. The authors also provide evidence that the XBP1u form attenuates XBP1s action (and hence UPR activation) by targeting it for degradation. Collectively, the authors have proposed an SPP regulated mechanism of UPR tuning through degradation of XBP1s via XBP1u.

The findings presented by Chen et al. reveal several novel aspects regarding the relationship between ERAD and UPR regulation. They demonstrate the presence of Derlin1 in a 500kD complex

with SPP and Trc8 that also binds XBP1u. They now provide even more data to convincingly show that XBP1u can adopt the conformation of a type II membrane protein, for which the hydrophobic sequence responsible is cleaved by SPP. A role for Derlin1-mediated recognition of the XBP1u ectodomain is also clearly demonstrated. Overall the revised manuscript is well written, with the additional data welcomed and effectively integrated. Overall, this work has been significantly improved and represents an important set of observations in the field of ER stress regulation.

RESPONSE TO REBUTTAL

Overall, the authors have basically addressed most of the points this reviewer has raised during the first round of evaluation.

1. In response to the question of Derlin1-SPP interaction discrepancy with other published work, the authors have satisfactorily explained their differences.

2. In response to concerns of the XBP1u putative topology, the authors have now convincingly demonstrated the topology of XBP1u using four independent assays.

3. In response to the query of endogenous XBP1u orientation, the lack of available reagents is unfortunate but understandable and the absence of any conclusion on this point does not adversely affect the conclusions drawn from this study.

4. With respect to ubiquitination of XBP1u, the authors have made some effort to demonstrate this point, however the banding patterns of the Ub western blots (particularly E4B) are not overly convincing. I would recommend that if an additional sample of XBP1u IP treated with epoxymycin was treated with a recombinant deubiquitinating enzyme (e.g. Usp2cc) and resulted in a disappearance of the HMW bands, it would go a long way to making this point more convincing.

5. The authors have now included fluorescence protease protection assays on the XPP1u mutants to confirm their orientation.

6. Regarding the apparent inconsistency of XPB1u fragment levels, the argument for limiting amounts due to post-lysis degradation could make detection difficult I suppose.

7. In response to the issue of lack of difference in XBP1u co-IP with SPP-WT and -DA, the authors have now included a quantification demonstrating a slightly increased level of interaction with the dominant-negative SPP, as would be expected. The added discussion regarding enzymatic processing rates of SPP is also welcomed.

8. With respect to the unidentifiable bands in Figure 7A & B, the argument that they are beyond the scope of this manuscript is satisfactory.

9. To address the concern of ectodomain specificity, the authors inclusion of additional date using chimeras with CD74 helped to clarify this point and helped to demonstrate the sufficiency of the XBP1u C-terminal tail.

10. To address the concern of assembly of the Derlin1-G170V mutant, the BN-PAGE included is sufficient.

11. The query into the presence of Derlin1 in the SPP-Trc8 complex with the XPB1u mutant, the BN-PAGE figure in E7F clearly demonstrate its presence, even though the temporal aspects are not resolved.

MINOR COMMENTS

Most of the comments from the original review have been satisfactorily addressed. However, on pg. 4, Trc8 interaction with SPP was not shown directly by Christianson et al. 2011 and so should not be cited.

2nd Revision - authors' response 22 August 2014

Response to the reviewer's comments

Referee #1:

… I am still puzzled about the extent by which XBP1u cleavage occurs by SPP. Is cleavage by SPP only a minor fraction of total XBP1u cleavage (in which case the whole story would be of less general interest)? Or does SPP mediate a large fraction of total XBP1u cleavage, making it a finding of wider interest. Fig 2G suggests that the SPP inhibitors strongly stabilize the endogenous XBP1u, which would argue for a more central role of SPP in XBP1u cleavage. In contrast the newly added Figure E4D suggests that a stabilization (inhibition of cleavage) of this tagged XBP1u only occurs with epoxomycin (proteasome inhibitor), but not with the SPP inhibitors, suggesting predominant proteasome cleavage.

Response: This is a fair point. Although we think part of the discrepancy is caused by a general higher inhibitor potency observed for short-term treatments (used for the chase assays), we realized that our L-685,458 stocks had gone bad. We repeated the steady-state analysis shown in Fig. E4D with fresh inhibitor lots now also limiting the incubation time (as indicated in the figure legend, see Expanded View Information page 4) and observe stabilization of XBP1u by both SPP inhibitors. Consistent with this increased potency, in the epoxomicin-SPP inhibitor double treated cells we now observe an even more pronounced suppression of cleavage fragments. Of note, single treatment with SPP inhibitors caused still a less pronounced accumulation of full-length XBP1u as compared to epoxomicin-treated cells, however, this is consistent with previously described differences in the potency of these compounds (Weihofen et al., 2003 JBC; Bland et al. 2003, JBC). Over all we provide several lines of evidence that the predominant pool of XBP1u is degraded by the concerted action of SPP and the proteasome.

Referee #2:

4. With respect to ubiquitination of XBP1u, the authors have made some effort to demonstrate this point, however the banding patterns of the Ub western blots (particularly E4B) are not overly convincing. I would recommend that if an additional sample of XBP1u IP treated with epoxymycin was treated with a recombinant deubiquitinating enzyme (e.g. Usp2cc) and resulted in a disappearance of the HMW bands, it would go a long way to making this point more convincing.

Response: Detection of ubiquitin modification is not always straightforward. Since ubiquitination of XBP1u has previously been shown by co-expression of His-tagged ubiquitin (Lee et al., 2003 PNAS), we followed a more stringent but slightly less sensitive approach to detect endogenous ubiquitin (that does not cause any potential overexpression artifacts). Although the referee's suggestion sounds intriguing, unfortunately using recombinant Usp2cc (BostonBiochem, Cat. # E-504) we have not been able to establish such an assay. We think it is fair to say that this approach so far is not standard in the field. To our knowledge Usp2cc has only been used by few proteomic approaches relying on enrichment protocols and highly sensitive MS detection (e.g. Stes et al., 2014 Journal of Proteome Research). Despite several attempts to establish Usp2cc treatment with total

cell lysates and immunoisolated material, we have not found a suitable assay window that allows isolation of ubiquitin conjugates and selective release by the recombinant enzyme. Despite the lack of an ultimate in vitro validation, we think that our data indicates that XBP1u is ubiquitinated prior proteasomal degradation. In order to sharpen our claim and to avoid any potential overstatement, we rephrased the relevant sentence (on page 14) that now reads: "… upon western analysis of immunoisolated XBP1u we detected several ubiquitinated species (Fig. E4B). This is consistent with a previous report showing that XBP1u is polyubiquitinated in HeLa cells (Lee et al., 2003)." Since a full description of the role ubiquitin plays is beyond the scope of our manuscript, we indicate (on page 22) that our model "remains speculative until the precise role of TRC8 in the SPP reaction cycle has been elucidated."

MINOR COMMENTS: … on pg. 4, Trc8 interaction with SPP was not shown directly by Christianson et al. 2011 and so should not be cited.

Response: We did not intend to refer here only to TRC8 as an SPP interaction partner – instead we aimed to name it as one example and refer to all studies that addressed how SPP interacts with the ER proteostasis network. In order to clarify this issue we now also list a second SPP interaction partner, UBAC2, a validated hit from the Christianson interaction study. The relevant sentence (on page 4) now reads: "It has been shown that SPP interacts with several quality control factors including the rhomboid pseudoprotease UBAC2 and the E3 ubiquitin ligase TRC8 (Christianson et al., 2011; Lee et al., 2010; Stagg et al., 2009)…".

