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Manuscript EMBOR-2015-40955

A conformational RNA zipper promotes intron-ejection during non-conventional XBP1 mRNA splicing

Jirka Peschek, Diego Acosta-Alvear, Aaron S. Mendez and Peter Walter

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Review timeline:

Submission date:	01 July 2015
Editorial Decision:	05 August 2015
Revision received:	31 August 2015
Editorial Decision:	07 September 2015
Revision received:	17 September 2015
Accepted:	18 September 2015

Editor: Esther Schnapp

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 05 August 2015

Thank you very much for the submission of your manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your study and I am pasting them below for your information. Since my colleague Esther Schnapp, who originally handled your manuscript, is currently out of the office, I am making a decision on her behalf to avoid any unnecessary delays.

You will see that all reviewers appreciate the interest of your findings and are, in principle, very supportive of publication of your study in our journal. They do, however, also raise some points that need further clarifications and/or discussions. Please see the attached reports for the details of these issues.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Given the relatively minor concerns raised by the referees I do, however, not anticipate that this will be a problem.

One additional point I would like to raise: We are in the process of updating the way in which we display additional/supplementary information. In essence, all supplementary figures are now called

Expanded View Figures and should be labeled and referenced as Figure EV1, Figure EV2 etc. in the main text of the manuscript. The legends for the EV figures should be incorporated in the main body of the text after the legends for the main figures. Please modify your additional figures accordingly. Please also note that we can only accommodate 5 expanded view figures (currently, your manuscript contains 6, and you might add more during the revision). Could you maybe identify one EV figure that could be incorporated into the manuscript as a main, i.e. regular, figure, so that in the end there are no more than 5 EV figures?

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS

Referee #1:

The manuscript by Peschek, Acosta-Alvear et al. is concise, clearly written and the conclusions are well-supported by the data and supplementary data provided. The methods are well described and the figures are clearly labeled.

Comments:

Figure 3a and 3c (lanes 8-14)
There is a dramatic difference in the amount of ligation between RTCB and Trl1. Perhaps a titration of ligase(s) would be informative.

With respect to both the in vitro and in vivo splicing reactions, the products of ligation are demonstrated by TBE-Urea PAGE (figure 3), multiplex PCR (4a) and immunoblot of translated protein (4b). It is important to understand the exact nature of the splice site ligation by directly sequencing the spliced products to confirm the site of cleavage by IRE1 and subsequent ligation by either Trl1 or RTCB.

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Figure 1b (line 82 in the text): what do the authors mean with "... the bifurcation-proximal part of this helix, which can invariably be extended"? Could this be supplied as a supplemental data or be further explained in the text? I also suggest writing Homo sapiens at the bottom, as they do in Suppl. Figure 1b.

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Suppl. Figure 2: Panel d) UAU (in violet, 845-847) should be UAC. In fact, this is actually one of three possible structures, as shown much later in the manuscript, in Suppl. Figure 6. See for example that structure 2 is already zipped. Why do the authors restrict their analysis to a single structure throughout the text? Is it due to the shape analysis in Suppl. Figure 2c-d? I suggest addressing this issue at the Discussion and when dealing with Suppl. Figure 6. In addition, it would be great to include some experimental data supporting the predicted ES1 secondary structure of the XBP1s mRNA. The same goes for the melting temperature of XBP1s and delta G of their predicted structure, just for comparison.

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In the text, when dealing with Figure 2b-d, please direct the reader to Figure 1a for the G to A substitution in bold, or Figure 1b with G in red.

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EMBO reports, Peschek et al.
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The unfolded-protein response (UPR) represents a signalling pathway that senses misfolded proteins in the ER and transduces the signal to the nucleus. This conserved response involves an unconventional splicing reaction of the XBP1 mRNA, which yields the XBP1 transcription factor, thereby inducing a gene expression program, which results in boosting the overall protein folding activity in the ER. This short manuscript focusses on the XBP1 splicing reaction (requiring the IRE1 endonuclease and a tRNA ligase), in particular the pathway of this non-conventional splicing mechanism, which is explained by a conserved RNA-structural rearrangement ("RNA unzippering"). Data are presented that support an intron ejection mechanism, based on RNA and secondary structure conservation (Figure 1), in vitro reactions combined with splice mutant analysis [Figures 2/3; cleavage/ligation; with both mammalian and yeast ligases, including a structure-destabilizing "NZ, non-zippering") mutant], in vivo evidence (Figure 4, based on a reporter construct and the NZ mutant).
Experiments are cleverly designed and controlled, very concisely described, data themselves and their presentation of consistent high quality. I can recommend publication.

Minor comments and questions:
1. In addition to the destabilizing mutant analyzed here ("NZ"), have the authors tested the effect of a hyperstabilizing mutation? Based on the secondary structure model, this seems at least possible to design.

2. As speculated in the Discussion, did the authors try to test in the in vitro system their model that IRE1 assemblies larger than a dimer are necessary for the intron cleavage reaction?
3. Figures 2/3: It may help the reader if the structure symbols explaining the intermediates and products of the in vitro reaction (Figure 2) were labeled in addition ("spliced, upstream intermediate", etc.), as to some extent done in Figure 3.

1st Revision - authors' response 31 August 2015

Detailed responses to each one of the Referees' concerns

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The difference in the amount of ligation by RTCB and Trl1 can be explained by (1) the source of the ligases we used for the splicing assays, and (2) the biochemical mechanism employed by either Trl1 or RTCB. Trl1 is a single recombinant protein expressed in *E. coli,* is highly pure and was concentrated prior to use. On the other hand, the RTCB-containing complex is immunopurified directly from cells expressing epitope-tagged RTCB. Thus, the material we employed is heterogeneous (RTCB being but one component of the complex), and not as highly concentrated as the recombinant yeast protein. In all of our experiments we looked at end-point products, and we have not derived any kinetic parameters because the ligation reactions are not directly comparable (*i.e.* the ligases employ different biochemical mechanisms, are obtained from different sources, and while the RTCB complex is multi-component in nature, Trl1 harbors all enzymatic activities in a single protein). For these reasons, we consider that a titration of the ligases will not add meaningful additional information or challenge our conclusions.

We do agree with the Referee, however, that direct sequencing of the ligation products helps to further clarify the nature of the splicing reaction. We have included these data in Expanded View Figure 3d. While we for yeast *HAC1* mRNA spliced in mammalian cells (Niwa et al., Cell 1999; PMID: 10619423) and the lab of Kazutoshi Mori for *XBP1* mRNA (Yoshida et al., Cell 2001; PMID 11779464) has confirmed the intron-exon junctions by sequencing analysis using RNA extracted from cells, to our surprise and to the best of our knowledge, sequence verification of the splicing reaction products carried out *in vitro* was never done.

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In line 82 of the text, we meant to state that the stem 1 (S1) of the bifurcate structure could be extended invariably among phylogenetically distant species. We have made corrections to the text to indicate this in lines 81-84 and have included these data in Expanded View Figure 1C.

Legend for Figure 1c: Typo: the intron IS colored in grey.

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The UAU sequence is the correct sequence for the transcript of mouse origin. Panels c and d of Supplemental Figure 2 show icSHAPE data for mouse Xbp1, while panels A/B show PARS data for human *XBP1*. We emphasize that the transcripts come from different species by showing different base-position numbering corresponding to each species in panels B and D and have included the species name below the corresponding structures for further clarification. We apologize if this was not sufficiently clear.

The structure in Supplemental Figure 2 is indeed one of three predicted possible structures. The reasons why we restricted the analysis to this main structure are the following: (1) both SHAPE and PARS analyses provide independent evidence for its existence in living cells, and (2) RNAse T1 digestion mapping performed in both the wild-type XBP1 transcript and our non-zipper mutant, which is predicted to assume a single conformation, points out to the same conclusion: the existence of a predominant structure. We have included these additional data in Expanded View Figure 4C and commented in lines 172-174 of the revised manuscript.

We agree with the Referee that some structural analysis of the predicted ES1 secondary structure is desirable. We have included additional data in the Expanded View Figure 3C and Expanded View Figure 4E. In particular, we have conducted an RNase T1 mapping of the spliced structure (Figure EV3C) and find it conforms to our prediction. We have also performed a melting curve analysis of the spliced transcript (Figure EV4C) and commented on lines 207-209 of the revised manuscript. Our results show that this RNA possesses a higher melting temperature than its unspliced counterpart, further substantiating the existence of a thermodynamically stable fold after splicing. The melt curve analysis is also consistent with the faster than expected mobility of the spliced RNA in TBE-urea PAGE gels. This observation is also in line with the spliced RNA being only partially denatured during TBE-urea PAGE electrophoresis. We have revised the discussion addressing these results in lines 256-264 of the revised manuscript.

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Good point: We have replaced “probe” by “transcript” throughout the manuscript in the revised version.

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In the text, when dealing with Figure 2b-d, please direct the reader to Figure 1a for the G to A substitution in bold, or Figure 1b with G in red.

We have addressed this issue and clarified it in the figure legend of Figure 2. We have also added a new figure (Fig. 2H) with a pictogram key to facilitate the interpretation of the gels.

Figures 3a & 3c: All reactions executed by RTCB could be boosted by its multiple turnover co-factor Archease. Now, while Archease seems to be absent in the figures, the authors have readily expressed and purified it... Did they use it? Where? Results shouldn't change, but the efficiency of the reaction might reach levels of Trl1 (see Figure 3, bottom panels). When comparing Trl1 and RTCB (Figure 3c), Trl1 is apparently much more sensitive to the mutations introduce to generate NZ.

All the reactions shown in Figure 3 were performed using immunopurified RTCB in the presence of recombinant archease for the reason stated by the Referee. We have clarified this point in the manuscript. We have made changes to the figure legend to address this point. In trial runs we performed, ligation with immunoprecipitated RTCB in the presence of archease did not reached the same levels reached by Trl1. We surmise this is because of intrinsic biochemical differences in the ligation reaction (see our responses to Referee #1 above).

Figure 3d: why not trying RTCB, perhaps as a Suppl. Figure?

We agree with the Referee that this is informative. We have performed the native PAGE gel experiment with RTCB and included those data as Expanded View Figure 4D and commented in lines 191-196 of the revised manuscript.

Figure 4d: I would not claim that the un-spliced form accumulates in the NZ mutant. The band is obviously present at time 0 and converted into the spliced form at a much lower rate. Also see that variations in GAPDH call for quantitation of the bands. However, and more importantly, why is the reporter already expressed in its spliced form even in the absence of UPR stress in lane 1? It is also clear that the expression of the NZ mutant in lanes 5 and 6 is not that severely compromised; this is a bit overstated in the text. Also, it would be better to have the same time points in figures 4b and 4d to better compare mRNA splicing levels and XBP1s expression.

We note that there is indeed an accumulation of the product encoded by the unspliced version of the reporter when compared to the wild-type control in Figure 4D. This accumulation is the product of a reduced conversion rate of unspliced into spliced substrate, brought about by the introduction of the non-zipper mutations. Thus, both our original interpretations as well as the argument raised by the Reviewer are valid. We have changed the narrative to address this apparent dichotomy (see lines 224-234 in the revised manuscript). While we agree that a quantification of the immunoreactive bands would be a befitting addition, in our view, it will not challenge our conclusion that introduction of the non-zipper mutation into the XBP1 transcripts severely compromises splicing.

With regards to the presence of spliced product in the absence of ER stress, this is a common observation stemming from both (1) the constitutive steady-state levels of XBP1 splicing observed in non-stressed cells (e.g., see PMID: 17612490) and (2) the long half-life of the reporter fusion protein, which is attributable to both the long half-life of GFP^{venus} and the removal of degradation signals from wild-type XBP1 (PMID: 16461360). Moreover, the handling of the cells in culture for this experiment (*i.e.* transfection with lipoplexes) generates mild ER stress. Combined, the aforementioned scenarios lead to the accumulation of the product encoded by spliced reporter construct well before the addition of chemical ER stressors.

Lastly, with respect to the different time series employed in RT-PCR and Western blot experiments, we decided to offset the timing for both experiments to best illustrate the phenotype on accumulation of the encoded protein. The maximum of the ER stress response in HEK293T cells treated with the ER stressors tunicamycin or thapsigargin is typically observed at around 4 hours (PMIDS: 17991856, 20798350). Thus, we consider that while having the same time series for both

RT-PCR and Western blot would make a congruous addition, it would not add information critical for the interpretation of the results.

Suppl. Figure 6: Besides discussing and considering these alternative structures, I suggest providing the distance of the splice sites. Could it be that one of these can be spliced by a single IRE1 dimer? Structure 2 looks like the splice sites are more closely together; also this structure resembles much more the final ES1 form.

We have provided measurements for the distances between the scissile bonds for the alternative structures in Expanded View Figure 5. Note that (1) these vary dramatically between the canonical structure (structure 1) and structures 2 and 3, and that (2), invariably, one loop in one of the two stem-loops is no longer a 7-mer, and hence, it is not a preferred substrate for IRE1. While a single-dimer cleavage at a single site on the RNA would be possible, the coordinated cleavage of both sites, followed by the zippering of the exons, which is a pre-requisite to complete the splicing reaction, is unlikely to occur. We have revised the discussion and included new comments on lines 274-278.

Discussion:
Line 231: Failure to form ES1 did not abrogate, but partially impaired the splicing, as Figure 4d shows.

We agree with the reviewer in that our mutant impairs splicing but does not abrogate it *in vivo. In vitro*, however, the phenotype is abrogation (when we reconstituted the reaction with Trl1) or compromised splicing (when we used RTCB). In the manuscript, we have addressed this (line 248 in the revised manuscript) and explained that while the mutations indeed disrupt the formation of ES1, splicing by RTCB could be completed, albeit inefficiently, because of the presence of accessory factors that may aid in intron removal (lines 176-178 in original submission, lines 179-181 in the revised manuscript).

Taken together, the paper needs some adjustments and possibly some extra data that could be presented in supplementary figures. It is essentially a solid manuscript describing a key RNA conformational change upon cleavage that ensures exon joining by the tRNA ligase.

Referee #3:

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The unfolded-protein response (UPR) represents a signalling pathway that senses misfolded proteins in the ER and transduces the signal to the nucleus. This conserved response involves an unconventional splicing reaction of the XBP1 mRNA, which yields the XBP1 transcription factor, thereby inducing a gene expression program, which results in boosting the overall protein folding activity in the ER. This short manuscript focuses on the XBP1 splicing reaction (requiring the IRE1 endonuclease and a tRNA ligase), in particular the pathway of this non-conventional splicing mechanism, which is explained by a conserved RNA-structural rearrangement ("RNA unzippering"). Data are presented that support an intron ejection mechanism, based on RNA and secondary structure conservation (Figure 1), in vitro reactions combined with splice mutant analysis [Figures 2/3; cleavage/ligation; with both mammalian and yeast ligases, including a structure-destabilizing "NZ, non-zippering") mutant], in vivo evidence (Figure 4, based on a reporter construct and the NZ mutant).
Experiments are cleverly designed and controlled, very concisely described, data themselves and their presentation of consistent high quality. I can recommend publication.

Minor comments and questions:

1. In addition to the destabilizing mutant analyzed here ("NZ"), have the authors tested the effect of a hyperstabilizing mutation? Based on the secondary structure model, this seems at least possible to design.

Unfortunately, a hyperstabilizing mutation is not compatible with the structure. All newly formed base-pairs within ES1, with the exception of a single U-A pair at the beginning of the extension, are already C-G pairs, so one cannot further stabilize ES1 without introducing mutations that would significantly alter the secondary structure of unspliced XBP1 transcript (*e.g.* removal of the 5'-UGAGU bulge of ES1).

2. As speculated in the Discussion, did the authors try to test in the in vitro system their model that IRE1 assemblies larger than a dimer are necessary for the intron cleavage reaction?

We have not tested in our *in vitro* experiments whether IRE1 assemblies larger than a dimer are necessary for the coordinated cleavage of the splice sites. However, extensive work from our lab both *in vitro* and *in vivo* (see PMIDs: 19079236, 20798350, 25986605), suggests that clusters larger than a dimer exist and are indeed required for full activation of the protein in yeast and mammals. We have indicated this strong correlation in our work. Furthermore, higher-order oligomers may not be obligatorily needed for coordinated cleavage by IRE1 *in vitro* since the reaction could, in principle, be completed by two independent IRE1 dimers. We have clarified this notion in lines 272-278 of the revised manuscript Discussion.

3. Figures 2/3: It may help the reader if the structure symbols explaining the intermediates and products of the in vitro reaction (Figure 2) were labeled in addition ("spliced, upstream intermediate", etc.), as to some extent done in Figure 3.

We have added a new figure (Fig. 2H) with a pictogram key to facilitate the interpretation of the gels.

2nd Editorial Decision 07 September 2015

Thank you for the submission of your revised manuscript to our journal. Given that referee 2 is satisfied with the revisions and supports its publication, we can in principle accept your manuscript now.

However, I noticed that the manuscript has 4 main figures but is laid out as a normal article and not a scientific report. My colleague Barbara may have missed to mention this in her decision letter. Scientific reports can have up to 5 figures, and full articles should have more than 5 figures. Given your 4 main figures and the conceptually short story, we should publish your manuscript as a scientific report. Therefore, you need to shorten the manuscript text to less than 35.000 characters including spaces, references and figure legends. You need to combine the Results and Discussion sections for a report, which will help to eliminate some redundancy that is inevitable when discussing the same experiments twice. You can also move commonly used Materials and Methods to the Appendix file, but please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file. I am sorry for this late notice regarding our two formats.

I am looking forward to receiving a shortened manuscript. Please let me know if yo have any questions.

REFEREE REPORTS

Referee #2:

I am satisfied with the authors' response to my comments.
I support publication in EMBO Reports.

2nd Revision - authors' response 17 September 2015

We are pleased to know that we have satisfied the concerns of the Reviewers and that our work is acceptable for publication in EMBO Reports. With regards to the formatting of our manuscript, and in agreement with your recommendations and those of Bernd Pulverer, we would like to avoid having to re-write the paper once more. We feel strongly that shortening the text would significantly weaken its accessibility to a wider readership.

Instead, we have integrated additional data that we had formerly placed on Expanded View Figures into five main figures. We've also scanned the manuscript again for redundancies or unnecessary repetitions and think that it reads well and to the point.

We have also made amendments to our "Response to the Reviewers" to reflect those changes, (*i.e.* new figure arrangement/altered figure numbering) and marked them in boldface type. We have included this modified Response to the Reviewers in our submission alongside. We also designed a cover image for your consideration.

Amended detailed responses to each one of the Referees' concerns

Referee #1:

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We do agree with the Referee, however, that direct sequencing of the ligation products helps to further clarify the nature of the splicing reaction. We have included these data in **Expanded View Figure 3C**. While we for yeast *HAC1* mRNA spliced in mammalian cells (Niwa et al., Cell 1999;

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We agree with the Referee that some structural analysis of the predicted ES1 secondary structure is desirable. We have included additional data in the **Expanded View Figure 3B and Expanded View Figure 3D**. In particular, we have conducted an RNase T1 mapping of the spliced structure (**Figure EV3B**) and find it conforms to our prediction. We have also performed a melting curve analysis of the spliced transcript (**Figure EV3D**) and **commented in lines 208-210** of the revised manuscript.

Our results show that this RNA possesses a higher melting temperature than its unspliced counterpart, further substantiating the existence of a thermodynamically stable fold after splicing. The melt curve analysis is also consistent with the faster than expected mobility of the spliced RNA in TBE-urea PAGE gels. This observation is also in line with the spliced RNA being only partially denatured during TBE-urea PAGE electrophoresis. We have revised the discussion **addressing these results in lines 256-264** of the revised manuscript.

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Good point: We have replaced “probe” by “transcript” throughout the manuscript in the revised version.

In the text, when dealing with Figure 2b-d, please direct the reader to Figure 1a for the G to A substitution in bold, or Figure 1b with G in red.

We have addressed this issue and clarified it in the figure legend of Figure 2. We have also added a new figure (Fig. 2H) with a pictogram key to facilitate the interpretation of the gels.

Figures 3a & 3c: All reactions executed by RTCB could be boosted by its multiple turnover co-factor Archease. Now, while Archease seems to be absent in the figures, the authors have readily expressed and purified it... Did they use it? Where? Results shouldn't change, but the efficiency of the reaction might reach levels of Trl1 (see Figure 3, bottom panels). When comparing Trl1 and RTCB (Figure 3c), Trl1 is apparently much more sensitive to the mutations introduce to generate NZ.

All the reactions shown in **Figures 3 and 4** were performed using immunopurified RTCB in the presence of recombinant archease for the reason stated by the Referee. We have clarified this point in the manuscript. We have made changes to the figure legends to address this point. In trial runs we performed, ligation with immunoprecipitated RTCB in the presence of archease did not reached the same levels reached by Trl1. We surmise this is because of intrinsic biochemical differences in the ligation reaction (see our responses to Referee #1 above).

Figure 3d: why not trying RTCB, perhaps as a Suppl. Figure?

We agree with the Referee that this is informative. We have performed the native PAGE gel experiment with RTCB and included those data as **Figure 4F** and commented **in lines 191-196** of the revised manuscript.

Figure 4d: I would not claim that the un-spliced form accumulates in the NZ mutant. The band is obviously present at time 0 and converted into the spliced form at a much lower rate. Also see that variations in GAPDH call for quantitation of the bands. However, and more importantly, why is the reporter already expressed in its spliced form even in the absence of UPR stress in lane 1? It is also clear that the expression of the NZ mutant in lanes 5 and 6 is not that severely compromised; this is a bit overstated in the text. Also, it would be better to have the same time points in figures 4b and 4d to better compare mRNA splicing levels and XBP1s expression.

We note that there is indeed an accumulation of the product encoded by the unspliced version of the reporter when compared to the wild-type control in **Figure 5D**. This accumulation is the product of a reduced conversion rate of unspliced into spliced substrate, brought about by the introduction of the non-zipper mutations. Thus, both our original interpretations as well as the argument raised by the Reviewer are valid. We have changed the narrative to address this apparent dichotomy (**see lines 223-234** in the revised manuscript). While we agree that a quantification of the immunoreactive bands would be a befitting addition, in our view, it will not challenge our conclusion that introduction of the non-zipper mutation into the XBP1 transcripts severely compromises splicing.

With regards to the presence of spliced product in the absence of ER stress, this is a common observation stemming from both (1) the constitutive steady-state levels of XBP1 splicing observed in non-stressed cells (e.g., see PMID: 17612490) and (2) the long half-life of the reporter fusion protein, which is attributable to both the long half-life of GFP^{venus} and the removal of degradation signals from wild-type XBP1 (PMID: 16461360). Moreover, the handling of the cells in culture for this experiment (*i.e.* transfection with lipoplexes) generates mild ER stress. Combined, the aforementioned scenarios lead to the accumulation of the product encoded by spliced reporter construct well before the addition of chemical ER stressors.

Lastly, with respect to the different time series employed in RT-PCR and Western blot experiments, we decided to offset the timing for both experiments to best illustrate the phenotype on accumulation of the encoded protein. The maximum of the ER stress response in HEK293T cells treated with the ER stressors tunicamycin or thapsigargin is typically observed at around 4 hours (PMIDS: 17991856, 20798350). Thus, we consider that while having the same time series for both RT-PCR and Western blot would make a congruous addition, it would not add information critical for the interpretation of the results.

Suppl. Figure 6: Besides discussing and considering these alternative structures, I suggest providing the distance of the splice sites. Could it be that one of these can be spliced by a single IRE1 dimer? Structure 2 looks like the splice sites are more closely together; also this structure resembles much more the final ES1 form.

We have provided measurements for the distances between the scissile bonds for the alternative structures in Expanded View Figure 5. Note that (1) these vary dramatically between the canonical structure (structure 1) and structures 2 and 3, and that (2), invariably, one loop in one of the two stem-loops is no longer a 7-mer, and hence, it is not a preferred substrate for IRE1. While a single-dimer cleavage at a single site on the RNA would be possible, the coordinated cleavage of both sites, followed by the zippering of the exons, which is a pre-requisite to complete the splicing reaction, is unlikely to occur. We have revised the discussion and included new comments **in lines 272-278.**

Discussion:
Line 231: Failure to form ES1 did not abrogate, but partially impaired the splicing, as Figure 4d shows.

We agree with the reviewer in that our mutant impairs splicing but does not abrogate it *in vivo. In vitro*, however, the phenotype is abrogation (when we reconstituted the reaction with Trl1) or compromised splicing (when we used RTCB). In the manuscript, we have addressed this (**line 248** in the revised manuscript) and explained that while the mutations indeed disrupt the formation of ES1, splicing by RTCB could be completed, albeit inefficiently, because of the presence of accessory factors that may aid in intron removal (lines 176-178 in original submission, **lines 178-181** in the revised manuscript).

Taken together, the paper needs some adjustments and possibly some extra data that could be presented in supplementary figures. It is essentially a solid manuscript describing a key RNA conformational change upon cleavage that ensures exon joining by the tRNA ligase.

Referee #3:

EMBO reports, Peschek et al.
"A conformational RNA zipper promotes intron-ejection during non-conventional XBP1 mRNA splicing"

The unfolded-protein response (UPR) represents a signalling pathway that senses misfolded proteins in the ER and transduces the signal to the nucleus. This conserved response involves an

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unconventional splicing reaction of the XBP1 mRNA, which yields the XBP1 transcription factor, thereby inducing a gene expression program, which results in boosting the overall protein folding activity in the ER. This short manuscript focuses on the XBP1 splicing reaction (requiring the IRE1 endonuclease and a tRNA ligase), in particular the pathway of this non-conventional splicing mechanism, which is explained by a conserved RNA-structural rearrangement ("RNA unzippering"). Data are presented that support an intron ejection mechanism, based on RNA and secondary structure conservation (Figure 1), in vitro reactions combined with splice mutant analysis [Figures 2/3; cleavage/ligation; with both mammalian and yeast ligases, including a structure-destabilizing "NZ, non-zippering") mutant], in vivo evidence (Figure 4, based on a reporter construct and the NZ mutant).

Experiments are cleverly designed and controlled, very concisely described, data themselves and their presentation of consistent high quality. I can recommend publication.

Minor comments and questions:

1. In addition to the destabilizing mutant analyzed here ("NZ"), have the authors tested the effect of a hyperstabilizing mutation? Based on the secondary structure model, this seems at least possible to design.

Unfortunately, a hyperstabilizing mutation is not compatible with the structure. All newly formed base-pairs within ES1, with the exception of a single U-A pair at the beginning of the extension, are already C-G pairs, so one cannot further stabilize ES1 without introducing mutations that would significantly alter the secondary structure of unspliced XBP1 transcript (*e.g.* removal of the 5'-UGAGU bulge of ES1).

2. As speculated in the Discussion, did the authors try to test in the in vitro system their model that IRE1 assemblies larger than a dimer are necessary for the intron cleavage reaction?

We have not tested in our *in vitro* experiments whether IRE1 assemblies larger than a dimer are necessary for the coordinated cleavage of the splice sites. However, extensive work from our lab both *in vitro* and *in vivo* (see PMIDs: 19079236, 20798350, 25986605), suggests that clusters larger than a dimer exist and are indeed required for full activation of the protein in yeast and mammals. We have indicated this strong correlation in our work. Furthermore, higher-order oligomers may not be obligatorily needed for coordinated cleavage by IRE1 *in vitro* since the reaction could, in principle, be completed by two independent IRE1 dimers. We have clarified this notion **in lines 270-278** of the revised manuscript Discussion.

3. Figures 2/3: It may help the reader if the structure symbols explaining the intermediates and products of the in vitro reaction (Figure 2) were labeled in addition ("spliced, upstream intermediate", etc.), as to some extent done in Figure 3.

We have added a new figure (Fig. 2H) with a pictogram key to facilitate the interpretation of the gels.

3rd Editorial Decision 18 September 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.