Stress sensor Ire1 deploys a divergent transcriptional program in response to lipid bilayer stress

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Re: JCB manuscript #201909165

Dr. Guillaume Thibault  
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60 Nanyang Drive, room 03n-28  
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Dear Dr. Thibault,

Thank you for submitting your manuscript entitled "ER stress sensor Ire1 deploys divergent transcriptional program in response to lipid bilayer stress". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all of the reviewers think that your study addresses an important and outstanding question regarding the molecular nature of how the unfolded protein response responds to lipid bilayer stress. However, the critical point was raised that additional experimental evidence is needed to support the main conclusion of the study - that there are two independent transcriptional responses for unfolded proteins and membrane stress. In particular, the reviewer 2 questioned the validity of data supporting the specificity of the responses and raised the related concern that the Ire1 alleles used in the study may not cleanly separate membrane and proteotoxic stresses as interpreted. A more complete kinetic analysis as suggested by the reviewer seems appropriate. However, we feel that reviewer 2's request for experiments that address the mechanistic basis of how different transcriptional programs are conducted is beyond the scope of the study. It may be possible to address issues regarding the involvement of RIDD raised by reviewers 1 and 2 with textual changes/additional discussion. The remaining reviewer comments all seem relatively minor, but reasonable, and will improve your study, and therefore should be addressed in your revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.
Reviewer #1 (Comments to the Authors (Required)):

The importance of the unfolded protein response (UPR) signaling pathway for maintaining the homeostasis of the proteotoxic and lipid bilayer stress (LBS)/lipotoxic stress of the endoplasmic reticulum (ER) has been well described. Components of the UPR pathway and the molecular mechanisms by which the UPR components respond to ER proteotoxic stress have been extensively investigated over the past several decades. However, the detailed molecular nature of LBS and the mechanisms by which UPR components respond to LBS have remained unclear. The work presented in this manuscript tackles important questions and provides a missing piece of the ER stress response mechanism.

Using both yeast and C. elegans experimental systems, Nurulain et al. performed genetic screens to
identify genes that induce LBS in cells with IRE1-LD, IRE1 that lacks the ER luminal domain. Researchers have performed similar screens previously, but the use of IRE1-LD is unique and enables the identification of genes that contribute to LBS. Moreover, the authors took the positive hits from the yeast screen and tested the homologous genes in C. elegans. These screens successfully identified genes that contribute to LBS levels or ER membrane integrity in both yeast and C. elegans, and included genes involved in the phosphatidylcholine (PC) biosynthetic pathway, vacuolar H+-ATPase genes, and metabolic genes.

The authors further investigated one of the genes identified, opi3, to dissect the nature of the LBS by examining both wild-type (WT) and IRE1-LD responses. Interestingly, the authors identified an IRE1 residue - located at the interface of the amphipathic helix and the transmembrane domain - that plays a critical role in LBS sensing. While the molecular nature of LBS itself remains to be determined, by analyzing genome-wide transcription responses the authors found that LBS can induce an LBS-specific transcription response distinct from the response of ER proteotoxic stress. Specific genes, including PIR3 and PUT1, were identified as HAC1-independent non-canonical UPR target genes. This is the first clear demonstration that IRE1 exerts two independent transcriptional outcomes depending upon the type of ER stress.

Overall, the experiments were well designed, executed, and included detailed analysis. In addition, some of the conclusions of the manuscript open new doors into our understanding of one of the missing links of the UPR mechanism. While most data are in agreement with the authors' conclusions, additional experiments and modifications of the data presentation would strengthen the conclusions. Specific comments are provided below:

#1. The results of the genes identified from the yeast screen are shown in Figure 1. While the authors aimed to provide a comprehensive representation of the findings, the presentation is rather confusing and the outcomes are difficult to grasp. In addition, the data shown in the supplemental tables aren't helpful. This is a pity as a lot of interesting information is probably hidden within the results, especially because the screen was performed with both full-length IRE1 and IRE1-LD. In the current format, it is difficult to decipher what findings are in agreement with previous results and what findings are new (or different) for IRE1-LD.

#2. Some critical details are missing in terms of how the data were analyzed. For example, Figure 2B shows results of C. elegans carrying hsp-4p::GFP UPR reporter exposed to RNAi bacteria for a specific gene for either 48 or 72 hours. How did the authors quantify the level of GFP in each animal? Based on the pictures shown, the GFP signals were not uniform within an individual worm. Presumably, this is because the reporter expression may differ in different tissues or because of potential heterogeneities between the worms exposed to the same RNAi. The heat maps shown in Figure 2C and Supplemental Table S2 do not seem to describe how the colors are assigned, beyond the description of weak, moderate, or strong. How are "strong", "moderate", and "weak" defined? How were the GFP levels assigned? How were the primary values (quantitated GFP levels) converted into color-coded data in Supplemental Table S2, and how was the heat map shown in Figure 2C generated? While Supplemental Table S2 contains a lot of data, some important values seem to be missing. Moreover, the non-essential information might be crowding the critical data.

#3. The authors also mentioned in the text that genes such as fat-4, fat-6, and fat-7, represent the most robust inducers. Given the heat map shown in Figure 2C, other genes appear to be more robust inducers. Additionally, the authors stated that the reporter expression of the worms fed RNAi bacteria for hmg-1, lpin-1, and vha-4 were partial rescued by the addition of choline (p5, lines
However, the primary data do not seem to be consistent with this conclusion.

#4. The authors conclude (p6 lane 167-168) that 35 of 36 hits are likely inducing the UPR without dramatically altering PC levels based on the choline supplementation rescue experiment. As this is an important point that could distinguish this study from previous ones, the authors should measure PC/PE levels in, at least, a few of the 35 hits.

#5. In the subcellular fractionation experiments in Figure S2B that characterize both IRE1-HA and IRE1ΔLD-HA, IRE1-HA appears in the supernatant fraction (S). This is present only in IRE1-HA cells but not IRE1ΔLD-HA cells. Why is IRE1-HA (but not IRE1ΔLD-HA) in the supernatant fraction? Furthermore, the mobility of IRE1-HA in (S) seems to differ between total and pellet fractions. Does this represent a posttranslational modification (such as N-glycosylation)?

#6. In previous studies, others have proposed that the oligomerization status of IRE1 might affect the ability of IRE1 to cleave HAC1/XBP1 introns versus engaging in RIDD. The authors should test RIDD with IRE1ΔLD in LBS-inducing knockout cells or in cells that activate both HAC1 splicing and LBS.

#7. In Figure 3E, the authors stated that "the over expression (OE) of KAR2 did not reduce the UPR". Based on the results for UPR activation caused by OE of KAR2/BiP in opi3 cells - the last two lanes in the graph - the observed difference appears rather small. This could result from heterogeneity of OE levels of KAR2 in each cell. Alternatively, this might be due to an experimental limitation, since KAR2 is a rather abundant protein, making it hard to achieve significant OE. The authors should perform Western blots or immunofluorescence to demonstrate levels of KAR2 expression in this experiment. Could they perform this experiment using a chemical chaperone to try to get clearer differences.

#8. In Figure 3B and 4C, HAC1 splicing levels should be quantified. Levels of the HAC1 splicing in ire1opi3 cells seem to differ between WT IRE1 and 1ΔLD in Figure 3B, but they are similar in Figure 4C.

In Figure 4C, the levels of HAC1u in inositol-depleted Ire1 (carrying ire1opi3) cells differ significantly from those in Ire1ΔLD. However, HAC1u in inositol-depleted Ire1 or Ire1ΔLD carrying ire1opi3 cells are similarly low, and addition of choline restores levels of HAC1u. What are the bases for these differences? Did a portion of HAC1u RNA undergo RIDD?

#9. In Figure 4A-C and Figure S2G, the split Venus experiment for examining IRE1 dimer/oligomer formation is a good experiment. In Figure S2G, Ire1ΔLD-VN/Ire1ΔLD-VC in scj1, and Figure 4A for Ire1ΔLD-VN/Ire1ΔLD-VC in ire1opi3 cells with carrier, there seem to be some level of Venus expression. The extent of the blue color for the lack of the Venus expression seen in Ire1ΔLD-VN/Ire1ΔLD-VC in ire1 cells (either carrier or DTT) differs from those of Ire1ΔLD-VN/Ire1ΔLD-VC in ire1opi3 cells.

Similarly, in Figure 4B and 4D some panels with Ire1ΔLD-VN/Ire1ΔLD-VC appear to have small foci. Unfortunately, the resolution of the image does not allow magnification to confirm this point. The authors should provide higher-resolution images.
Reviewer #2 (Comments to the Authors (Required)):

Review on 'ER stress sensor Ire1 deploy a divergent transcriptional program in response to lipid bilayer stress' by Ho et al..

The present study addresses the unfolded protein response (UPR) in yeast and worms and studies the contribution of lipid bilayer stress and proteotoxic stress. The authors generated a deletion variant of Ire1 that lacks an ER luminal domain and thus cannot sense unfolded proteins in the lumen of the ER. They perform screens in yeast and worms to identify genes that predominantly activate Ire1 via lipid bilayer stress. They identify a critical residue in Ire1 from yeast, which is crucial to activate the UPR when the ratio of PC and PE lipids is perturbed. Transcriptome analyses show significant changes of the cellular transcriptome caused by different forms ER stress.

The study is potentially interesting for the broad readership of JCB and provides interesting data, but it is suffering from several weaknesses outlined below. Given that the main claims of the manuscript remain hypothetical and are not strongly supported by the data, I cannot recommend this manuscript for publication at JCB at this stage. I recommend the authors to perform additional experiments and to consider alternative scenarios to rationalize their data.

Main points:

1) One of the main weakness of this study is that the timing of DTT-induced ER stress and lipid bilayer stress is not considered. The activity of the UPR in DTT-stressed cells is measured after relatively short DTT-treatments, while lipid bilayer stress caused by a perturbed PC-to-PE ratio is prolonged. The authors should consider to perform additional experiments with prolonged forms of proteotoxic stress to allow for a fair comparison. Quite possibly, the main difference causing the distinct transcriptomes reflect a different timing of ER stress and a distinct degree of UPR activation.

2) What if DTT-induced stress and lipid bilayer stress would jointly contribute to the stability of oligomeric forms of Ire1. Deletion of the entire luminal domain is likely to lower the sensitivity to lipid bilayer stress and in fact this is also suggested by their data. The authors should provide a more thorough discussion on how two types of stress would affect the activity of Ire1 and provide experimental data to distinguish alternative scenarios. What is the impact of duration of ER stress? What is the impact of prolonged, low ER stress versus short, intense ER stress?

3) The description of two different transcriptional programs caused by two forms of ER stress is potentially interesting. In its current form, the manuscript does not provide any reliable indication for how this distinct transcriptional program would be generated. It seems that the oligomeric state (monomers, dimers, higher oligomers) contribute to this distinction, but the assays to assays the oligomeric state have caveats (see below).

In fact, the main conclusion of the manuscript given by the title is not fully supported by the data. a) There is no evidence for how the transcription factor HAC1 should mediate two different signaling events. Is there are structural change in HAC1 or is it just the level of HAC1 that determines the transcriptional program? What is the impact of prolonged UPR activation?
b) The conditions of lipid bilayer stress a used in this study might perturb other signaling pathways in the cells. How can the authors exclude that lipid bilayer stress affects the availability of UPREs and the entire transcriptome predominantly by indirect means?
c) While the dysfunctional response to lipid bilayer stress of an IRE1 mutant (R537Q) is taken as an indication for a conformational change during lipid bilayer stress, there is not attempt to experimentally validate this. The authors should provide more experimental evidence for how different forms of ER-stress could result in different signals. They should discuss more extensively, how different durations and intensities of ER stress could result in similar results as observed.

4) The finding that a single mutation R537Q in Ire1 renders the protein insensitive to a perturbed PC-to-PE ratio is very interesting. Does this mutation also impair the response to lipid bilayer stress caused by inositol-depletion or by the deletion of MGA2 (regulating phospholipid saturation)?

5) The authors use full length Ire1 and IRE1ΔLD lacking the luminal domain, but they do not comment on the expression level. Given that the activity of Ire1 is controlled by oligomerization, even a low degree of overexpression can lead to a different sensitivity of the system.

6) The deletion of the entire ER luminal domain is quite drastic and may have a larger impact on the oligomerization and activation of Ire1 (also during lipid bilayer stress). In fact, Figure 3B, 3C, 4C show that IRE1 lacking the LD is less efficient than full length IRE1 in generating spliced HAC1 and signaling an UPR. This means that not only the sensitivity to proteotoxic stress, but also the sensitivity to LBS is disturbed by the loss of the LD domain. Therefore, the IRE1ΔLD is not a good control for the transcriptomic analysis. The authors should provide more data to test impact of LBS and proteotoxic stress on the transcriptome. A helpful control would be a regulated expression of HAC1 from a regulated promotor (to simulate prolonged ER stress). Another important control would be a mutant variant of full length IRE1 where both ER-luminal dimerization and oligomerization interfaces have been disrupted by mutation.

Thus, the authors should discuss to what extent their phenotypic and transcriptomic data may represent a perturbed response of the IRE1ΔLD variant to lipid bilayer stress. The data provide evidence that the luminal domain contributes to the activation of IRE1 during lipid bilayer stress, presumably by increasing the stability of oligomeric species of IRE1. This should be clearly discussed.

In general, I find it hard to draw mechanistic, molecular conclusions on the signaling events that lead to IRE1 downstream signaling based on transcriptomic data using the IRE1ΔLD variant. It is conceivable that the distinct transcriptome of IRE1ΔLD-expressing cells is at least in part due to the different dynamic behavior of oligomeric IRE1 species and less so due to a conformational change, which is specific to conditions of lipid bilayer stress.

7) The overlap and integration of screening data from S. cerevisiae and C. elegans are very low. This is somewhat disappointing, but also interesting. Can the authors discuss potential reasons for this outcome?

8) In Figure S2A the authors test the localization of IRE1ΔLD versus IRE1-HA. It seems that IRE1ΔLD localizes quite strongly to the nuclear envelope, while Ire1-HA is found throughout the entire ER. The authors should comment on this possible mislocalization and the possible implications. A particularly critical caveat is that C-terminally tagged IRE1 is not functional!

9) The overexpression experiments (with a soluble, luminal domain of IRE1 and KAR2) shown in
Figure 3D and 3E and discussed in line 229-247 are performed with cells, which were perturbed by many different means: lipid bilayer stress, tunicamycin, and overexpression. These manipulations are quite complex and do not allow drawing mechanistic conclusions from it. I would remove/adjust this section.

10) (see also point 8) The authors use C-terminally tagged variants of Ire1 (split-venus versions and tagged HA-tagged variants). C-terminally tagged IRE1 is not functional. Unless the authors can show that these variants of Ire1 can mediate a response to proteotoxic or LBS in ire1Δ cells, I do not think that the oligomeric state of the non-active variant is very informative.

11) It should be noted that split-venus approach has a remarkable caveat: it connects proteins irreversibly. It is hard to discuss the role of protein dynamics for signaling based on these data.

12) How does RIDD contribute to the response to lipid bilayer stress versus DTT? The possibility of RIDD activity should be discussed more prominently.

General statement: Instead of claiming that Ire1 undergoes a non-defined structural change that leads to a non-defined difference in the structure of HAC1 and - as a consequence - to a distinct set HAC1-regulated target genes, an alternative (more likely model) would say that different durations and degrees of UPR activation (as judged by HAC1 splicing) can lead to different snapshots of the transcriptome. However, the contribution of RIDD would have to be discussed in this context as well.

The authors should state that it remains to be shown whether the observed, distinct transcriptomes are caused by specific differences between proteotoxic stresses and LBS, or if they rather reflect a combination of indirect effects (from LBS activating other signaling pathways) and specific differences between prolonged and acute forms of ER-stress.

Minor issues:

line 24-26: The authors state that unresolved, chronic ER stress leads to apoptosis. They should mention that S. cerevisiae lacks an ER stress-induced apoptosis to avoid confusions.

line 40-41: Membrane fluidity has not been firmly implicated in the activation of the UPR as a mechanistic cause. The authors should mention 'perturbed bilayer properties' or 'aberrant membrane stiffening' to avoid misinterpretation.

Line 46-47 and Line 186-188: The authors should state even more clearly that both a too high and a too low PC-to-PE ratio has been implicated in ER stress and UPR activation.

line 86: The 'To address this gap' does not become clear to me. Is there a missing sentence?

line 103-108: I do not understand what the authors mean with this section. Does it mean that both variants of Ire1 (with and without luminal domain) can sense proteotoxic stress? Does it mean that STE24, SPC2, SCJ1 and GET1 cause lipid bilayer stress even though they have known functions in protein folding? While the text says 'both query strains are able to report UPR activation through proteotoxic and LBS' line 117-118 says that the 'query strain IRE1ΔLD is insensitive to proteotoxic stress.' A clarification is required.
The reader may not understand in this section why it is noteworthy that peroxisomal genes have not been identified in the overlap of query strains. A clarification would help.

The authors correctly state that loss of OLE1 is lethal in yeast. However, they are fatty acid autotrophs and it is clear that a downregulation of fatty acid desaturation activates the UPR by causing lipid bilayer stress (PMID: 23891562).

It is unclear, what is meant by 'rotational orientations'.

The statement that PE promotes negative membrane curvature is wrong. Negative intrinsic curvature would be correct.

The meaning of this sentence does not become clear to me: 'Binding affinity of Ire1' for what? How would binding by Ire1 change membrane stiffening? A clarification would help.

The authors seek to gain a better understanding of the unfolded protein response (UPR) when it is induced by membrane perturbations. The UPR was originally identified as a response to the buildup of unfolded peptides in the ER lumen. However, in the last decade, several groups demonstrated that UPR sensors can alternatively be activated by a range of imbalances in the lipid composition of the ER membrane, which the authors collectively refer to as lipid bilayer stress (LBS). Activation of the UPR by LBS remains incompletely understood and constitutes an exciting research direction.

The manuscript presents the following key new pieces of information: (1) Systematic identification of genes whose depletion activates the UPR sensor Ire1 via changes in the ER membrane; (2) Identification of a single residue in Ire1's juxtamembrane region that is required for Ire1's response to LBS but not protein folding stress; and (3) Mapping of the differences in transcriptional responses between the LBS and the "conventional" misfolded protein-induced UPR. These findings are novel, well supported by data, and of broad interest to the field.

Concerns:

1. In the section titled "LBS-activated Ire1 induces the UPR independently of its oligomeric state" and the related Figure 4, authors need to carefully revise the text to distinguish between high-order oligomerization and dimerization. As currently written, the terms are used nearly interchangeably, painting an inaccurate picture of the experiment. The observed Ire1 puncta are indicative only of large oligomers and not dimers, as single Venus fluorophores would not be detectable in this assay. The BiFC approach does not work well for low-abundance proteins and has the additional caveat of providing extra affinity to dimerization-prone proteins via the split-FP fragments. The only conclusion that can be safely drawn from Fig. 4A is that LBS does not drive Ire1 into large oligomers - one really cannot conclude anything about dimerization. In fact, given Ire1's low expression levels, this experiment may be more convincing with a bright monomeric FP such as mNeonGreen instead of a split Venus.
2. The limited C. elegans screen (Fig. 2) is one of the weakest parts of the paper. It does not add much to the story, and there is minimal direct overlap between the genes identified in worms and those identified in yeast. The fact that "genes in several pathways resulted in robust UPR induction across species", including "several metabolic genes" (lines 178-180) is of questionable relevance since only metabolism-related genes were selected for screening in worms in the first place. Furthermore, visual screening of GFP fluorescence intensities is a very error-prone method, and even the example images in Fig. 2B raise questions. For instance, tag-335 is annotated as having "strong" intensity while pri-2 is "weak", but as the data are presented, there are no obvious differences between the two. This figure should either be strengthened by quantitative analysis, be relegated to the supplement, or be omitted. In either case, the figure would greatly benefit from annotation of key pathways, rather than just showing a list of gene names and expecting the reader to know or look up what any of them are.

3. Data in Figure 6, while interesting, are overinterpreted. The authors correctly note that the transcriptional profile of Δopi3 cells will include a number of genes that are regulated independently of the UPR. Deletion of HAC1 or IRE1 is impossible due to their synthetic lethality. However, the presented workaround is complex and unsatisfying. Would it be possible to chemically induce LBS, e.g. by inositol depletion, and use those cells (with and without full-length or ΔLD Ire1) as additional controls? It is of concern that many, if not most, of the identified genes in the Δopi3 cells have little to do with Ire1 or the UPR and are rather hallmarks of PC deficiency.

4. The manuscript would significantly benefit from careful copy editing. The manuscript contains numerous typos, along with awkward or grammatically incorrect sentences, that distract the reader and make the authors' intended messages hard to grasp.

In conclusion, the manuscript is novel, tackles an interesting topic, and is worthy of publication provided that the concerns listed above are addressed.

Signed: Vladislav Belyy and Peter Walter

PS: After perusing the other reviewers' comments, we have nothing to add or modify.
February 26th, 2020

Jodi Nunnari, PhD
Editor-in-Chief
Journal of Cell Biology

Dear Prof. Jodi Nunnari,

Please find enclosed our revised manuscript entitled “ER stress sensor Ire1 deploys a divergent transcriptional program in response to lipid bilayer stress” (JCB #201909165).

We are grateful for your efforts and those of the reviewers in evaluating our original submission. We considered the reviewer’s comments carefully and incorporated major changes to the manuscript to address their concerns, including several new experiments. Of note, we have improved the analysis and visual representation of our yeast and C. elegans screens and further validated some hits in C. elegans. To address the consensus concern of the three reviewers in using split Venus to monitor Ire1 clusters, we acquired new fluorescence images using the well-established insertion of mNeonGreen within Ire1 kinase domain (Ire1-mNG). The new data agree with and strongly support our findings using split Venus – namely that Ire1 cluster formation during proteotoxic and LBS stress are different. We have also performed new experiments to quantify UPR target gene expression during chronic ER stress, and the results further support our model whereby the UPR program diverges in response to lipid bilayer stress. Furthermore, we have extensively rewritten the manuscript to improve its clarity.

Below, we outline our responses and actions, point-by-point, to reviewer comments. With those changes, we believe that the manuscript is substantially improved and ready for publication in the Journal of Cell Biology.

We thank you for your efforts.

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REMARKS TO AUTHORS

Please note that most changes are highlighted in red throughout the revised manuscript.

Reviewer #1 comments

1. The results of the genes identified from the yeast screen are shown in Figure 1. While the authors aimed to provide a comprehensive representation of the findings, the presentation is rather confusing and the outcomes are difficult to grasp. In addition, the data shown in the supplemental tables aren’t helpful. This is a pity as a lot of interesting information is probably hidden within the results, especially because the screen was performed with both full-length IRE1 and IRE1-ΔLD. In the current format, it is difficult to decipher what findings are in agreement with previous results and what findings are new (or different) for IRE1-ΔLD.

We thank the reviewer for the feedback. We have extensively modified Fig. 1. Of note, we have highlighted previously reported deletion strains (Jonikas et al., Science 2019; PMID 19325107) which activate the UPR through IRE1 with black circles in the revised Fig. 1C. In addition, we modified Table S1 with corresponding Fig. 1B colors to highlight the hits within each category.

New Figure 1:
2. Some critical details are missing in terms of how the data were analyzed. For example, Figure 2B shows results of C. elegans carrying hsp-4p::GFP UPR reporter exposed to RNAi bacteria for a specific gene for either 48 or 72 hours. How did the authors quantify the level of GFP in each animal? Based on the pictures shown, the GFP signals were not uniform within an individual worm. Presumably, this is because the reporter expression may differ in different tissues or because of potential heterogeneities between the worms exposed to the same RNAi. The heat maps shown in Figure 2C and Supplemental Table S2 do not seem to describe how the colors are assigned, beyond the description of weak, moderate, or strong. How are "strong", "moderate", and "weak" defined? How were the GFP levels assigned? How were the primary values (quantitated GFP levels) converted into color-coded data in Supplemental Table S2, and how was the heat map shown in Figure 2C generated? While Supplemental Table S2 contains a lot of data, some important values seem to be missing. Moreover, the non-essential information might be crowding the critical data.

AND

3. The authors also mentioned in the text that genes such as fat-4, fat-6, and fat-7, represent the most robust inducers. Given the heat map shown in Figure 2C, other genes appear to be more robust inducers. Additionally, the authors stated that the reporter expression of the worms fed RNAi bacteria for hmgs-1, lipn-1, and vha-4 were partial rescued by the addition of choline (p5, lines 164-166, revised manuscript p5, lines 152-154). However, the primary data do not seem to be consistent with this conclusion.

We appreciate the feedback and acknowledge that the screen and the scoring needed better explanation, description, and visualization. Furthermore, the data themselves needed quantification, which we have now performed and included (see also response to reviewer #3). Answers to the specific questions/comments are below. Table S2, Figure 2, and the results and methods text have been updated to better explain the screen and its results.

"How did the authors quantify the level of GFP in each animal?"

a. This screen, like many using C. elegans GFP reporters, was scored visually under a fluorescent dissection microscope. This way, we classified individual worms as “high”, “medium”, or “low” fluorescence emitting worms. The number of worms in each category was counted. This is now clearly indicated in Table S2 and explained in the methods (page 19, lines 526-548).

"Based on the pictures shown, the GFP signals were not uniform within an individual worm."

b. The reviewer is correct – like many C. elegans GFP-reporters, the widely used hsp-4p::gfp reporter displays intra- as well as inter-animal variability. This likely reflects both tissue-specific effects as well as RNAi efficiency that can vary between tissues and from animal to animal. For this reason, we scored relatively large numbers of animals at each scoring time point (usually n>30) and performed triplicate validation with the reporter after the duplicate screen.

“How are "strong", "moderate", and "weak" defined? How were the GFP levels assigned? How were the primary values (quantitated GFP levels) converted into color-coded data in Supplemental Table S2, and how was the heat map shown in Figure 2C generated?”

c. We appreciate the need to better explain these definitions and our scoring. In line with the request of reviewer #3, we have now used our visual scoring data (“high”, “medium”, and “low”, explained above) to generate a quantitative scoring rubric, as follows: “strong” hit if >70%/worms display strong or medium fluorescence; “moderate” hit if 10-69%/worms display strong or medium fluorescence; “weak” hit if any worms displayed fluorescence that exceeded that seen in the negative control. The classifications "strong", "moderate", and "weak" were then assigned values of 5, 3, and 1 in the screen and validation experiments, and these values were aggregated to generate summative scores. Visually, "strong", "moderate", and "weak" are expressed as dark, medium, and light green or red in Table S2 and in Fig. 2. To better explain these details, we have updated Table S2, Fig. 2 and its legend, and the methods (557-562) section.
**New Figure 2C:**

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48h  72h  48h  72h

x x x x
x x x x
x x x x
x x x x
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"...mentioned in the text that genes such as fat-4, fat-6, and fat-7 represent the most robust inducers. Given the heat map shown in Figure 2C, other genes appear to be more robust inducers."

d. While fat-6 is indeed one of our strongest hits, we agree that some other RNAi clones are more robust inducers than fat-4 and -7. Our goal here was not to pinpoint the strongest inducers, but to show that our screen identified genes previously known to induce the UPR-ER when inactivated by RNAi, an important validation finding. This includes strong hits fat-6, sams-1, and hmgs-1, and moderate hit fat-7. We have revised the text to better explain these findings (p5 lines 148-152).

"...the authors stated that the reporter expression of the worms fed RNAi bacteria for hmgs-1, lpin-1, and vha-4 were partial rescued by the addition of choline (p5, lines 164-166, revised manuscript p5, lines 152-154). However, the primary data do not seem to be consistent with this conclusion."

e. We agree that the data as previously presented did not effectively show that choline reduced the UPR-ER activation caused by some RNAi clones. To rectify this, we reanalyzed the choline rescue experiment data with our quantification method described above. This revised analysis shows that choline supplementation substantially weakens hsp-4 fluorescence in worms fed vha-4, pdi-1, hmgs-1, sams-1 (previously published, validating our data) or mdt-15 RNAi (please see Table S2, tab “Choline resc.”).

4. The authors conclude (p6 line 167-168, revised manuscript p5, line 154-155) that 35 of 36 hits are likely inducing the UPR without dramatically altering PC levels based on the choline supplementation rescue experiment. As this is an important point that could distinguish this study from previous ones, the authors should measure PC/PE levels in, at least, a few of the 35 hits.

We agree with the reviewer that it will be extremely interesting to study the effects of the newly identified RNAi clones on the *C. elegans* membrane lipidome. The PC/PE levels of mdt-15, sams-1, and fat-6 RNAi treated worms have been studied before and agree well with our proposed model (i.e. that choline supplementation is beneficial when PC is low). However, quantifying membrane PC (and other lipid species) for our hits is a long-term experiment that is beyond the scope of the present manuscript.

5. In the subcellular fractionation experiments in Figure S2B [updated as Fig. S3B] that characterize both IRE1-HA and IRE1ΔLD-HA, IRE1-HA appears in the supernatant fraction (S). This is present only in IRE1-HA cells but not IRE1ΔLD-HA cells. Why is IRE1-HA (but not IRE1ΔLD-HA) in the supernatant fraction? Furthermore, the mobility of IRE1-HA in (S) seems to differ between total and pellet fractions. Does this represent a posttranslational modification (such as N-glycosylation)?

We thank the reviewer to point this out. The lower bands appearing in total (T) and in the soluble (S) fractions are unspecific (denoted by an asterisk). The sentence "The asterisk indicates unspecific bands" has been added to Fig. S3B legend (previously Fig. S2B). To further clarify this important point, we have repeated the assay to separate Ire1-HA and Ire1ΔLD-HA on the same gel. The unspecific bands appear in total (T) and the soluble (S) fractions below Ire1-HA and above Ire1ΔLD-HA. We have updated the figure below (left). We include the empty vector on the right to further highlight the unspecific band (data not shown in the manuscript).
New Figure S3B:

6. In previous studies, others have proposed that the oligomerization status of IRE1 might affect the ability of IRE1 to cleave HAC1/XBP1 introns versus engaging in RIDD. The authors should test RIDD with IRE1ΔLD in LBS-inducing knockout cells or in cells that activate both HAC1 splicing and LBS.

AND

Reviewer #2, comment #12: How does RIDD contribute to the response to lipid bilayer stress versus DTT? The possibility of RIDD activity should be discussed more prominently.

We agree with the reviewers that it will be relevant to ask if IRE1α and IRE1ΔLDα RIDD activities and substrate specificity vary during proteotoxic stress and LBS in mammalian cells. However, S. cerevisiae Ire1 has no detectable RIDD activity according to Peter Walter’s lab (Li et al., Elife 2018; PMID 29985129). Although two potential RIDD substrates were previously identified using recombinant S. cerevisiae Ire1 (Tam et al., Cell Rep 2014; PMID 25437541), Peter Walter’s lab reported to be unable to reproduce this result. On the other hand, it should be noted that S. pombe Ire1 lacks an HAC1/XBP1 mRNA ortholog but instead it restores ER homeostasis through RIDD activity (Kimmig et al., Elife 2012; PMID 23066505). Considering that it is still unclear if Ire1 exhibits RIDD activity in S. cerevisiae, we feel that it is beyond the scope of this current manuscript to demonstrate if RIDD activity is (1) working in our hands and if (2) RIDD activity contributes to the response to lipid bilayer stress versus DTT.

7. In Figure 3E, the authors stated that "the over expression (OE) of KAR2 did not reduce the UPR". Based on the results for UPR activation caused by OE of KAR2/BiP in Δopi3 cells - the last two lanes in the graph - the observed difference appears rather small. This could result from heterogeneity of OE levels of KAR2 in each cell. Alternatively, this might be due to an experimental limitation, since KAR2 is a rather abundant protein, making it hard to achieve significant OE. The authors should perform Western blots or immunofluorescence to demonstrate levels of KAR2 expression in this experiment. Could they perform this experiment using a chemical chaperone to try to get clearer differences.

AND

Reviewer #2, comment #9: The overexpression experiments (with a soluble, luminal domain of IRE1 and KAR2) shown in Figure 3D and 3E and discussed in line 229-247 [revised manuscript lines 211-225] are performed with cells, which were perturbed by many different means: lipid bilayer stress, tunicamycin, and overexpression. These manipulations are quite complex and do not allow drawing mechanistic conclusions from it. I would remove/adjust this section.

To address the issue raised by both reviewers, we replaced OE KAR2 with the chemical chaperone 4-phenylbutyric acid (4-PBA). As previously reported, we added 1 mM 4-PBA to the media 15 minutes before 1h Tm incubation, when indicated (Mai et al., FEMS Yeast Res 2018; PMID 29452364). We monitored the UPR activation using the UPRE-LacZ reporter and the HAC1 mRNA splicing assay. As expected, 4-PBA attenuated Tm-induced UPR in IRE1 cells (Fig. 3, E and F). In opi3ΔIRE1 cells, 4-PBA failed to reduce the UPR. In contrast to a
previous report that 4-PBA accelerates Ire1 degradation (Mai et al., 2018), Ire1 protein levels were unchanged in IRE1 and opi3ΔIRE1 cells upon 4-PBA treatment (Fig. S3 D). These findings reinforce the notion that LBS is the main driver of the UPR in opi3Δ.

New Figure 3E and F:

E

F

New Figure S3D

8. In Figure 3B and 4C [updated as Fig. S4C], HAC1 splicing levels should be quantified. Levels of the HAC1 splicing in Δire1Δopi3 cells seem to differ between WT IRE1 and 1ΔLD in Figure 3B, but they are similar in Figure 4C.

In Figure 4C, the levels of HAC1u in inositol-depleted Ire1 (carrying ire1Δ) cells differ significantly from those in Ire1ΔLD. However, HAC1u in inositol-depleted Ire1 or Ire1ΔLD carrying ire1Δ opi3Δ cells are similarly low, and addition of choline restores levels of HAC1u. What are the bases for these differences? Did a portion of HAC1u RNA undergo RIDD?

We thank the reviewer for the suggestion. We have quantified the percentage of spliced HAC1 mRNA (HAC1s) to total HAC1 mRNA (spliced and unspliced HAC1 mRNA) of the triplicates. The numbers have been added below the gels of Fig. 3B, 4A, and S4C. As mentioned above to address the comment #6, S. cerevisiae Ire1 is unlikely to exhibit RIDD activity as previously reported by Peter Walter’s lab (Li et al., Elife 2018; PMID 29985129). We have updated the figures.

New Figure 3B:
New Figure 4A:

[Diagram of experiment results]

9. In Figure 4A-C and Figure S2G, the split Venus experiment for examining IRE1 dimer/oligomer formation is a good experiment. In Figure S2G, Ire1ΔLD-VN/Ire1ΔLD-VC in scj1Δ, and Figure 4A for Ire1ΔLD-VN/Ire1ΔLD-VC in ire1Δopi3Δ cells with carrier, there seem to be some level of Venus expression. The extent of the blue color for the lack of the Venus expression seen in Ire1ΔLD-VN/Ire1ΔLD-VC in ire1Δ cells (either carrier or DTT) differs from those of Ire1ΔLD-VN/Ire1ΔLD-VC in ire1Δopi3Δ cells.

Similarly, in Figure 4B and 4D some panels with Ire1ΔLD-VN/Ire1ΔLD-VC appear to have small foci. Unfortunately, the resolution of the image does not allow magnification to confirm this point. The authors should provide higher-resolution images.

AND

Reviewer #2 comment #10. The authors use C-terminally tagged variants of Ire1 (split-venus versions and tagged HA-tagged variants). C-terminally tagged IRE1 is not functional. Unless the authors can show that these variants of Ire1 can mediate a response to proteotoxic or LBS in ire1Δ cells, I do not think that the oligomeric state of the non-active variant is very informative.

AND

Reviewer #3 comment #1: In the section titled "LBS-activated Ire1 induces the UPR independently of its oligomeric state" and the related Figure 4, authors need to carefully revise the text to distinguish between high-order oligomerization and dimerization. As currently written, the terms are used nearly interchangeably, painting an inaccurate picture of the experiment. The observed Ire1 puncta are indicative only of large oligomers and not dimers, as single Venus fluorophores would not be detectable in this assay. The BiFC approach does not work well for low-abundance proteins and has the additional caveat of providing extra affinity to dimerization-prone proteins via the split-FP fragments. The only conclusion that can be safely drawn from Fig. 4A is that LBS does not drive Ire1 into large oligomers - one really cannot conclude anything about dimerization. In fact, given Ire1's low expression levels, this experiment may be more convincing with a bright monomeric FP such as mNeonGreen instead of a split Venus.

To address the consensus concern of the three reviewers in using split Venus to monitor clusters, we have inserted 3xHA tag with ymNeonGreen (Botman et al., Sci Rep 2019; PMID 30783202) between Ire1 Kinase domain residues I571 and G572 (Ire1-mNG) as previously reported (Aragón et al., Nature 2009; PMID 19079237). We observed the formation of clusters only in strains expressing Ire1-mNG upon DTT treatments (Fig. 4 and S3, E and F). This agrees with our findings using the split Venus fragments to monitor Ire1 clusters by bimolecular
fluorescence complementation assay (BiFC). Therefore, we thought it to be relevant to still report our BiFC data (Fig. S4). We have updated both the manuscript and the figures.

Updated manuscript (lines 232-248):

“To monitor Ire1 clusters, we inserted HA tag with ymNeonGreen between the Ire1 kinase domain residues I571 and G572 (Ire1-mNG), as previously reported (Aragon et al., 2009; Botman et al., 2019). To validate Ire1 variants, we assayed HAC1 mRNA splicing. Ire1-mNG was sufficient to induce HAC1S upon DTT treatment, inositol depletion, and in opI3Δ cells, while ire1ΔopI3Δ cells expressing Ire1ΔLD-mNG displayed HAC1S accumulation (Fig. 4 A). Ire1-mNG was uniformly distributed and colocalized with the ER marker dsRed-HDEL in ire1Δ cells (Fig. 4 B). As expected, we detected Ire1-mNG clusters as bright punctate structures at the ER upon DTT treatment. Unexpectedly, in ire1ΔopI3Δ cells, puncta were absent with both Ire1-mNG and Ire1ΔLD-mNG, while Ire1-mNG formed fewer puncta compared to ire1Δ cells in response to DTT. To assess this discrepancy with previous reports, LBS was induced with a 4 h inositol depletion in ire1Δ cells. Few puncta were detected with Ire1-mNG reporter but not with Ire1ΔLD-mNG (Fig. 4 C and S3 E). Consistent with the lack of clusters observed in opI3Δ cells, get1Δ, scj1Δ, and ste24Δ mutants showed the absence of fluorescent puncta (Fig. 4 D). As expected, clusters of Ire1 were evident in these mutants upon DTT treatment (Fig. S3 F). To further validate our findings, we used a pair of split Venus fragments to monitor Ire1 clusters by bimolecular fluorescence complementation assay (BiFC) (Robida and Kerppola, 2009). Clusters of Ire1 were observed only upon DTT treatment (Fig. S4). Together, these findings suggest that the formation of large Ire1 clusters is mostly driven by proteotoxic stress and that Ire1ΔLD is unable to form oligomers.”

New Figure 4:
New Figure S3, E and F:
Reviewer #2 comments

1. One of the main weaknesses of this study is that the timing of DTT-induced ER stress and lipid bilayer stress is not considered. The activity of the UPR in DTT-stressed cells is measured after relatively short DTT-treatments, while lipid bilayer stress caused by a perturbed PC-to-PE ratio is prolonged. The authors should consider to perform additional experiments with prolonged forms of proteotoxic stress to allow for a fair comparison. Quite possibly, the main difference causing the distinct transcriptomes reflect a different timing of ER stress and a distinct degree of UPR activation.

We agree with the reviewer that 1h Tm/DTT treatment and opi3Δ induce acute and chronic ER stress, respectively. Therefore, the comparison of the transcriptome induced by proteotoxic stress to LBS might differ by changing the Tm/DTT incubation time. The transcriptomic data in Fig. 6 was intended to provide a snapshot of different experimental conditions. To strengthen our hypothesis, we have carried out a ChIP assay (Fig. 6, E-K) to highlight the differential binding of Hac1 to the promoters of a subset of genes during proteotoxic stress and LBS. To further address the reviewer concerns, we have performed a 4 h treatment time course with 1 mM and 5 mM DTT (data not shown in the manuscript but reported below) as previously reported, to reach maximum UPR induction at both concentrations (Rubio et al., JCB 2011; PMID 21444684).

We opted to use 1 mM DTT as the response was similar at both concentrations of DTT. Next, we grew opi3ΔIRE1 cells under unstressed condition by supplementing the media with choline to maintain PC synthesis. To induce acute ER stress, we depleted the media of choline for 24 h. This assay agrees with our ChIP data that LBS leads to a divergent UPR program when compared to proteotoxic stress. We have updated Figure 6 and the manuscript.

Updated manuscript (lines 340-349):

“As the UPR is constitutively activated in opi3Δ cells (chronic ER stress), we grew opi3ΔIRE1 cells under unstressed condition by supplementing the media with choline to maintain PC synthesis followed by 24 h choline depletion to induce acute ER stress which is comparable to 4 h DTT treatment in IRE1 cells. Around 40% HAC1S was detected at 0.5 h and 12 h in IRE1 and opi3ΔIRE1 cells, respectively (Fig. 6 L). Concurrently, only the mRNA level of thiol oxidase ERO1 was significantly upregulated at 0.5 h compared to 0 h in DTT treated IRE1 cells while the mRNA levels of ERO1, PB2, and PIR3 were increased by 2.8, 3.8, and 14.5 fold, respectively, at 12 h compared to 0 h in opi3ΔIRE1 cells (Fig. 6 M). Furthermore, only ERO1 levels correlate with HAC1S levels in IRE1 cells whereas ERO1, PB2, and PIR3 levels resemble the increase of HAC1S in opi3ΔIRE1 cells. Together, with the ChiP data, these findings strongly argue for the existence of a specific UPR<sub>LBS</sub> transcriptional program.”

New Figure 6, L and M:
2. What if DTT-induced stress and lipid bilayer stress would jointly contribute to the stability of oligomeric forms of Ire1. Deletion of the entire luminal domain is likely to lower the sensitivity to lipid bilayer stress and in fact this is also suggested by their data. The authors should provide a more thorough discussion on how two types of stress would affect the activity of Ire1 and provide experimental data to distinguish alternative scenarios. What is the impact of duration of ER stress? What is the impact of prolonged, low ER stress versus short, intense ER stress?

As pointed out by the reviewer, Ire1 luminal domain has been shown to play a role in Ire1 oligomerization (Credle et al., PNAS 2005; PMID 16365312). Therefore, several strategies have been used to uncouple Ire1 sensing ER stress from LBS to proteotoxic stress. In yeast, Ire1 lacking the luminal domain residues 243 to 272 (Ire1ΔIII) has been shown to be unresponsive to proteotoxic stress (Tm and DTT) while being activated by LBS (inositol depletion) (Promlek et al., Mol Biol Cell 2011; PMID 21775630 and Halbleib et al., Mol Cell 2017; PMID 28689662). Thus, the truncation of the core stress-sensing region (ΔIII) abolishes the binding of unfolded protein while retaining the capacity to dimerize. In mammalian cells, most of the luminal domain of IRE1α was deleted (Δ29-442) to create the variant ΔLD-IRE1α (Volmer et al., PNAS 2013; PMID 23487760), which is effectively very similar to our yeast Ire1ΔLD variant. Using ΔLD-IRE1α, the authors demonstrated that it is activated by LBS [palmitic acid and stearoyl CoA desaturase 1 (SCD1) inhibitor] at a similar level to Ire1α WT (XBP1 mRNA splicing, IRE1α phosphorylation). To eliminate the possible unknown contributions of a truncated LD (ΔII), we opted to remove most of the LD.

Furthermore, we have addressed the impact of the duration of ER stress in comment #1 above.

3. The description of two different transcriptional programs caused by two forms of ER stress is potentially interesting. In its current form, the manuscript does not provide any reliable indication for how this distinct transcriptional program would be generated. It seems that the oligomeric state (monomers, dimers, higher oligomers) contribute to this distinction, but the assays to assay the oligomeric state have caveats (see below). In fact, the main conclusion of the manuscript given by the title is not fully supported by the data.

3a. There is no evidence for how the transcription factor HAC1 should mediate two different signaling events. Is there are structural change in HAC1 or is it just the level of HAC1 that determines the transcriptional program? What is the impact of prolonged UPR activation?

We agree with the reviewer that when Ire1 senses ER stress, the Hac1-mediated response is assumed to be linear until ER homeostasis is restored. However, there are several lines of evidence suggesting the contrary in yeast and mammals, particularly during LBS. Previously, we demonstrated that the UPR transcriptional program is modulated through differential target gene expression depending on the source of stress [Thibault et al., PNAS 2011; PMID 22143797]. The resulting differential UPR transcriptional program included the ablation of Hsp70 co-chaperone SCJ1 which was identified to induce UPR\textsuperscript{LBS} in our genome-wide genetic screen. In C. elegans, we recently demonstrated that the transcriptome diverges dramatically between UPR\textsuperscript{RT} and UPR\textsuperscript{LBS}, including hundreds of genes upregulated in an IRE-1-dependent (IRE1α homolog) manner during LBS [Koh et al., J Cell Sci 2018; PMID 30333136]. Similarly, in mammalian cells, the ATF6-modulated UPR program diverged upon LBS in comparison to proteotoxic stress [Tam et al., Dev Cell 2018; PMID 30086303]. In agreement with these findings, HAC1 mRNA level was revealed to be enhanced by a bipartite signal, misfolded proteins and either inositol depletion or temperature shift [Leber et al., PLoS Biol 2004; PMID 15314654]. In the presence of one of the latter signals in ire1Δ cells, the levels of HAC1 mRNA more than doubled, indicating an Ire1-independent mechanism maintaining protein quality control, mounting an alternative transcriptional program. Additionally, another group demonstrated the autoregulation of HAC1 during periods of extreme and prolonged ER stress by a positive feedback loop of Hac1 binding to its promoter [Ogawa et al., Genes Cells 2004; PMID 15009095]. There is a delicate balance faced by Ire1 in responding to the stress it encounters and a finely tuned response for the activation of particular genes to adapt to cellular changes [Leber et al., PLoS Biol 2004; PMID 15314654]. In addition to transcriptional regulation, transcription factor Hac1 is regulated by multiple factors at the protein level. Gcn4, a transcriptional activator of amino acid biosynthetic genes, works synergistically with Hac1 at the promoter of UPR target genes [Patil et al., PLoS Biol 2004 15314660]. In accordance with these findings, we identified and validated PBI2, PIR3, and PUT1, genes that are exclusively upregulated during UPR\textsuperscript{LBS} which supports the integration of multiple cellular stimuli to mount a divergent transcriptional response by Hac1. We hypothesize that additional unidentified trans-acting factors might regulate Hac1 transcriptional factor. It will be explored in future studies.

3b. The conditions of lipid bilayer stress a used in this study might perturb other signaling pathways in the cells. How can the authors exclude that lipid bilayer stress affects the availability of UPREs and the entire transcriptome predominantly by indirect means?
We agree with the reviewer that the conditions of LBS used in our study as well as alternative ways of perturbing the lipid bilayer used by different groups (inositol depletion or palmitic acid) might affect other cellular signaling pathways. Indeed, it is plausible that some UPR-independent cellular stress responses might modulate the UPR-mediated transcriptome through UPREs or Hac1. However, our findings are relevant in the context of metabolic diseases such as non-alcoholic fatty liver disease (NAFLD). As with our opi3Δ LBS model, disruption of phosphatidylcholine (PC) / phosphatidylethanolamines (PE) ratio is associated with ER stress and the UPR. Therefore, in the NAFLD context, it is highly plausible that LBS will remold the UPR-mediated transcriptome through the ER stress element (ERSE) or XBP1. Consequently, it will be important in the future to dissect the mechanism driving the synergy between UPR-independent cellular stress responses and the UPR transcriptome.

3c. While the dysfunctional response to lipid bilayer stress of an IRE1 mutant (R537Q) is taken as an indication for a conformational change during lipid bilayer stress, there is not attempt to experimentally validate this. The authors should provide more experimental evidence for how different forms of ER-stress could result in different signals. They should discuss more extensively, how different durations and intensities of ER stress could result in similar results as observed.

We agree with the reviewer that we have no experimental evidence validating our hypothesis that R537 contributes to Ire1 conformational change by LBS. Accordingly, we remove the sentence "The arginine residue located at the interface of the amphipathic and transmembrane helices of Ire1 is essential for LBS sensing, suggesting a conformational change inducing Ire1 activation (Fig. 5)" in the discussion (line 375).

4. The finding that a single mutation R537Q in Ire1 renders the protein insensitive to a perturbed PC-to-PE ratio is very interesting. Does this mutation also impair the response to lipid bilayer stress caused by inositol-depletion or by the deletion of MGA2 (regulating phospholipid saturation)?

We thank the reviewer for the suggestion. We monitored HAC1S mRNA in inositol-depleted IRE1(R537Q) and IRE1ΔLD(R537Q) cells. Upon inositol depletion, we observed the splicing of mRNA HAC1 in IRE1 and IRE1ΔLD cells (Fig. 5 E) with similar ratios to Fig. 4C. Interestingly, HAC1S was detected at a similar ratio in IRE1(R537Q) cells when compared to IRE1 cells upon inositol deletion. HAC1S was absent in IRE1ΔLD(R537Q) cells. Together, these finding suggests that inositol induce ER stress through proteotoxic stress and LBS. We have updated both the manuscript and the figure.

Updated manuscript (lines 271-273):

"Interestingly, HAC1S was undetected in IRE1ΔLD(R537Q) upon inositol depletion, suggesting that the lack of inositol induces proteotoxic stress and LBS (Fig. 5 E)."

New Figure 5E:

5. The authors use full length Ire1 and IRE1ΔLD lacking the luminal domain, but they do not comment on the expression level. Given that the activity of Ire1 is controlled by oligomerization, even a low degree of overexpression can lead to a different sensitivity of the system.

The expression levels of Ire1-HA and IRE1ΔLD-HA (ΔLD) are similar in the total fraction (T) of the alkaline carbonate assay in Fig. S3B (see below). Similarly, the expression levels of Ire1-mNG and ΔLD-mNG are similar in IRE1 and in opi3ΔIRE1 cells (please refer to Reviewer #1, comment #8 for an updated Fig. 4).

New Figure S3B:
6. The deletion of the entire ER luminal domain is quite drastic and may have a larger impact on the oligomerization and activation of Ire1. In fact, Figure 3B, 3C, 4C show that IRE1 lacking the LD is less efficient than full length IRE1 in generating spliced HAC1 and signaling an UPR. This means that not only the sensitivity to proteotoxic stress, but also the sensitivity to LBS is disturbed by the loss of the LD domain. Therefore, the IRE1ΔLD is not a good control for the transcriptomic analysis. The authors should provide more data to test impact of LBS and proteotoxic stress on the transcriptome. A helpful control would be a regulated expression of HAC1 from a regulated promotor (to simulate prolonged ER stress). Another important control would be a mutant variant of full length IRE1 where both ER-luminal dimerization and oligomerization interfaces have been disrupted by mutation.

As briefly discussed above (Reviewer #2, comment #2), different approaches have been reported to uncouple the UPR activation triggered by LBS from proteotoxic stress. In yeast, Ire1 lacking the luminal domain residues 243 to 272 (Ire1ΔIII) only responds to LBS (Promlek et al., Mol Biol Cell 2011; PMID 21775630 and Halbleib et al., Mol Cell 2017; PMID 28689662). In mammalian cells, most of the luminal domain of IRE1α was deleted (Δ29-442) to create the variant ΔLD-IRE1α (Volmer et al., PNAS 2013; PMID 23487760) which is very similar to our yeast Ire1ΔLD variant. Using ΔLD-IRE1α, the authors demonstrated that it is activated by LBS [palmitic acid and stearoyl CoA desaturase 1 (SCD1) inhibitor] at a similar level to IRE1α WT (XBP1 mRNA splicing, IRE1α phosphorylation). We opted to the latter approach. We reasoned that it will be best to remove the entire luminal domain as a large remaining luminal domain is likely to interact with molecular chaperones and other ER proteins that might play a role in Ire1ΔIII activation, deactivation, or inhibition.

We quantified the levels of spliced HAC1 mRNA (HAC1S). The levels of HAC1S in opi3Δ cells are ~70%, 140%, ~109%, and ~103% of the levels of opi3Δ/IRE1ΔLD cells in Fig. 3B, 4A, 5C, and S4C, respectively. Additionally, the UPR activation monitored with the reporter UPRE-GFP are almost identical in Δget1, Δscj1, Δspc2, and Δste24 when comparing Ire1ΔLD to Ire1 in Fig. S1, D and E.

We agree with the reviewer that HAC1 mRNA spliced levels are lower in opi3Δ cells when comparing Ire1ΔLD to Ire1 in Fig. 3B, but the levels are similar in Fig. 4C and 5C. HAC1 mRNA spliced monitored by qPCR are similar with Ire1ΔLD and Ire1 in Δopi3 cells. It should be noted that

Moreover, as suggested by the reviewer, we have collected more data on the regulation of Hac1 and target genes during LBS and proteotoxic stress. Please refer to Reviewer #2, comment #1.

Thus, the authors should discuss to what extent their phenotypic and transcriptomic data may represent a perturbed response of the IRE1ΔLD variant to lipid bilayer stress. The data provide evidence that the luminal domain contributes to the activation of IRE1 during lipid bilayer stress, presumably by increasing the stability of oligomeric species of IRE1. This should be clearly discussed.

In general, I find it hard to draw mechanistic, molecular conclusions on the signaling events that lead to IRE1 downstream signaling based on transcriptomic data using the IRE1ΔLD variant. It is conceivable that the distinct transcriptome of IRE1ΔLD-expressing cells is at least in part due to the different dynamic behavior of oligomeric IRE1 species and less so due to a conformational change, which is specific to conditions of lipid bilayer stress.

We have carried out the ChiP assay and the time-course qPCR using WT IRE1 in ire1Δ and opi3Δ to further validate our transcriptome data. Moreover, we have address some of the reviewer concerned in the comments following comments above: Reviewer #2, comments #1, 2, 3a, 3b, 3c, 5, and above within #6.
7. The overlap and integration of screening data from S. cerevisiae and C. elegans are very low. This is somewhat disappointing, but also interesting. Can the authors discuss potential reasons for this outcome?

We agree that these findings are somewhat surprising. To some extent, this likely reflects the fact that several genes whose inactivation results in UPR activation in C. elegans cause lethality in yeast when the corresponding ortholog is deleted. Moreover, while indeed few individual genes show conservation, yeast and worms share pathways and processes that, when inactivated or compromised, results in UPR-ER activation, including: Protein Disulfide Isomerase, FA desaturation, PC synthesis, Vacuolar H^+ATPase, sterol metabolism, and others.

8. In Figure S2A the authors test the localization of IRE1ΔLD versus IRE1-HA. It seems that IRE1ΔLD localizes quite strongly to the nuclear envelope, while Ire1-HA is found throughout the entire ER. The authors should comment on this possible mislocalization and the possible implications. A particularly critical caveat is that C-terminally tagged IRE1 is not functional!

As we have included numerous images of Ire1-mNG and Ire1ΔLD-mNG in Fig. 4, it is now clear that Ire1ΔLD-mNG nicely colocalize with the ER marker dsRed-HDEL. Please refer to Reviewer #1, comment #8 for an updated Fig. 4.

9. The overexpression experiments (with a soluble, luminal domain of IRE1 and KAR2) shown in Figure 3D and 3E and discussed in line 229-247 are performed with cells, which were perturbed by many different means: lipid bilayer stress, tunicamycin, and overexpression. These manipulations are quite complex and do not allow drawing mechanistic conclusions from it. I would remove/adjust this section.

We agree with the reviewer that our approach was not conclusive. As Reviewer #1 raised similar concerns, we invite Reviewer #2 to see our response to Reviewer #1, comment #7.

10. (see also point 8) The authors use C-terminally tagged variants of Ire1 (split-Venus versions and tagged HA-tagged variants). C-terminally tagged IRE1 is not functional. Unless the authors can show that these variants of Ire1 can mediate a response to proteotoxic or LBS in ire1Δ cells, I do not think that the oligomeric state of the non-active variant is very informative.

In yeast, Ire1 with a C-terminal HA or FLAG epitopes were previously reported to be functional (Kimata et al., J Cell Biol 2007; PMID 17923530 and Promlek et al., Mol Biol Cell 2011; PMID 21775630). To further validate the functionality of our C-terminally tagged variants of Ire1, we carried a spotting assay in the presence of Tm.

To monitor Ire1 oligomerization, we have replaced Ire1 split-Venus versions by a 3xHA tag with ymNeonGreen (Botman et al., Sci Rep 2019; PMID 30783202) between Ire1 Kinase domain residues I571 and G572 (Ire1-mNG) as previously published (Aragón et al., Nature 2009; PMID 19079237). We invite Reviewer #2 to see our response to Reviewer #1, comment #9 for more details. We have demonstrated the functionality of Ire1 and Ire1ΔLD variants with a C-terminal Venus tag by monitoring HAC1 mRNA splicing (HAC1^5).

Updated Figure S4C:
11. It should be noted that split-venus approach has a remarkable caveat: it connects proteins irreversibly. It is hard to discuss the role of protein dynamics for signaling based on these data.

We have addressed this concern by adding internal mNeonGreen to the cytosolic domain of Ire1 variants. As this issue was raised by the 3 reviewers, we invite Reviewer #2 to see our response to Reviewer #1, comment #9.

12. How does RIDD contribute to the response to lipid bilayer stress versus DTT? The possibility of RIDD activity should be discussed more prominently.

We thank the reviewer for the suggestion. As Reviewer #1 raised similar questions, we invite Reviewer #2 to see our response to Reviewer #1, comment #6.

General statement: Instead of claiming that Ire1 undergoes a non-defined structural change that leads to a non-defined difference in the structure of HAC1 and -as a consequence- to a distinct set HAC1-regulated target genes, an alternative (more likely model) would say that different durations and degrees of UPR activation (as judged by HAC1 splicing) can lead to different snapshots of the transcriptome. However, the contribution of RIDD would have to be discussed in this context as well.

The authors should state that it remains to be shown whether the observed, distinct transcriptomes are caused by specific differences between proteotoxic stresses and LBS, or if they rather reflect a combination of indirect effects (from LBS activating other signaling pathways) and specific differences between prolonged and acute forms of ER-stress.

We addressed the reviewer concerned by inducing acute ER stress in opi3Δ with a 24 h choline depletion. Please refer to Reviewer #2, comment #1.

Minor issues:

1. line 24-26: The authors state that unresolved, chronic ER stress leads to apoptosis. They should mention that S. cerevisiae lacks an ER stress-induced apoptosis to avoid confusions.

We have modified the introduction accordingly (line 27-29).

2. line 40-41: Membrane fluidity has not been firmly implicated in the activation of the UPR as a mechanistic cause. The authors should mention ‘perturbed bilayer properties’ or ‘aberrant membrane stiffening’ to avoid misinterpretation.

We have replaced the term “membrane fluidity” by “lipid bilayer properties” (line 39).

3. Line 46-47 and Line 186-188: The authors should state even more clearly that both a too high and a too low PC-to-PE ratio has been implicated in ER stress and UPR activation.

We have modified the sentence in lines 42-43 to include “too high or too low phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio” and in line 173 to include “Increased or decreased PC/PE ratios”.

4. line 86: The ‘To address this gap’ does not become clear to me. Is there a missing sentence?

We have replaced “To address this gap” with “To systematically identify cellular perturbations inducing LBS,” (line 78-79).

5. line 103-108: I do not understand what the authors mean with this section. Does it mean that both variants of Ire1 (with and without luminal domain) can sense proteotoxic stress? Does it mean that STE24, SPC2, SCJ1 and GET1 cause lipid bilayer stress even though they have different functions in protein folding? While the text says ‘both query strains are able to report UPR activation through proteotoxic and LBS’ line 117-118 says that the ‘query strain IRE1ΔLD is insensitive to proteotoxic stress.’ A clarification is required.

We have clarified the section (lines 89-99).

6. Line 140-141: The reader may not understand in this section why it is noteworthy that peroxisomal genes have not been identified in the overlap of query strains. A clarification would help.
We modified Figure 1 extensively (see Reviewer #1 comment #1). Therefore, peroxisomal genes are no longer highlighted in Figure 1. Thus, we removed the unclear statement related to peroxisomal genes.

7. Line 176-178: The authors correctly state that loss of OLE1 is lethal in yeast. However, they are fatty acid autotrophs and it is clear that a downregulation of fatty acid desaturation activates the UPR by causing lipid bilayer stress (PMID: 23891562).

We thank the reviewer for the suggestion. We have added the supporting information to the manuscript (lines 164-166).

8. Line 395-396: It is unclear, what is meant by 'rotational orientations'.

We agree with the reviewer that the sentence was unclear. We modified the sentence (lines 354-356).

9. Line 406-407: The statement that PE promotes negative membrane curvature is wrong. Negative intrinsic curvature would be correct.

We have modified the manuscript accordingly (line 365).

10. Line 412-414: The meaning of this sentence does not become clear to me: 'Binding affinity of Ire1' for what? How would binding by Ire1 change membrane stiffening? A clarification would help.

We agree with the reviewer that the sentence was unclear. We modified the sentence (lines 371-372).
**Reviewer #3 comments**

1. In the section titled "LBS-activated Ire1 induces the UPR independently of its oligomeric state" and the related Figure 4, authors need to carefully revise the text to distinguish between high-order oligomerization and dimerization. As currently written, the terms are used nearly interchangeably, painting an inaccurate picture of the experiment. The observed Ire1 puncta are indicative only of large oligomers and not dimers, as single Venus fluorophores would not be detectable in this assay. The BiFC approach does not work well for low-abundance proteins and has the additional caveat of providing extra affinity to dimerization-prone proteins via the split-FP fragments. The only conclusion that can be safely drawn from Fig. 4A is that LBS does not drive Ire1 into large oligomers - one really cannot conclude anything about dimerization. In fact, given Ire1’s low expression levels, this experiment may be more convincing with a bright monomeric FP such as mNeonGreen instead of a split Venus.

We thank the reviewer for this great suggestion. We have revised Fig. 4 where we replaced the BiFC approach with Ire1 containing an internal ymNeonGreen (mNG) tag. As the issue was raised by the 3 reviewers, we invite Reviewer #3 to see our response to Reviewer #1, comment #9.

2. The limited *C. elegans* screen (Fig. 2) is one of the weakest parts of the paper. It does not add much to the story, and there is minimal direct overlap between the genes identified in worms and those identified in yeast. The fact that "genes in several pathways resulted in robust UPR induction across species", including "several metabolic genes" (lines 178-180) is of questionable relevance since only metabolism-related genes were selected for screening in worms in the first place. Furthermore, visual screening of GFP fluorescence intensities is a very error-prone method, and even the example images in Fig. 2B raise questions. For instance, tag-335 is annotated as having "strong" intensity while pri-2 is "weak", but as the data are presented, there are no obvious differences between the two. This figure should either be strengthened by quantitative analysis, be relegated to the supplement, or be omitted. In either case, the figure would greatly benefit from annotation of key pathways, rather than just showing a list of gene names and expecting the reader to know or look up what any of them are.

We agree with the reviewer that it is not surprising that we identified several metabolic genes and have removed this text.

With regards to visual scoring of GFP in worms, this method is widely used in *C. elegans* due to its utility in detecting subtle changes and tissue-specific effects. However, we agree that quantitative data should provide additional value and provide that in this revision (please see also response to reviewer #1). Specifically, we used our existing visual scoring data (where worms were classified into emitting high, medium, or weak fluorescence) to generate a quantitative scoring rubric. We assigned the term "strong" hit if ≥70%/worms display strong or medium fluorescence; the term "moderate" hit if 10-69%/worms display strong or medium fluorescence; and the term "weak" hit if any worms displayed fluorescence that exceeded that seen in the negative control. The categories "strong", "moderate", and "weak" were then expressed as dark, medium, and light green or red in Table S2 and Fig 2. This new quantitative analysis led to a revised list of hits: clones R06C7.5/adsl-1, pri-2, T08H10.1; F18E2.1 were removed; vice versa, clones hmgr-1 (in agreement with our functionally related hit hmg-1) and let-754 emerged as new hits, bringing the total number of hits to 34 (please see Table S2 tab "Hits Fig 2"). We have updated the images in Fig 2 to better reflect the data in Table S2.

To corroborate our primary screen with additional quantitative data, we used real-time PCR to quantify the mRNA levels in wild-type worms (i.e., worms not containing the *hsp-4p::gfp* transgene). We studied two endogenous UPR genes: *hsp-4* itself, and Y41C4A.11, a coatomer protein complex subunit previously shown to be specifically induced by the IRE-1–XBP-1 branch of the UPR (Hou et al. 2014). We tested 15 RNAi clones at the 48-hour timepoint. Of these 15 clones, 5 were classified as moderate or strong hits at 48 hours in our *hsp-4p::gfp* screen; these 5 all significantly induced Y41C4A.11, and 4 of 5 significantly induced *hsp-4*. Vice versa, of the 15 tested clones, 6 were classified as non-hits at the 48 hrs timepoint in our *hsp-4p::gfp* screen (they were only scored as hits at 72 hours); of these 6, only 2 activated either *hsp-4* or Y41C4A.11, and none of the 6 activated both. This validates our hits, and shows that our *hsp-4p::gfp* screening strategy successfully identified novel UPR activating RNAi clones.

We agree that the Figure needed some better annotation of the hits and have organized them into functional categories as possible.

3. Data in Figure 6, while interesting, are overinterpreted. The authors correctly note that the transcriptional profile of ∆opi3 cells will include a number of genes that are regulated independently of the UPR. Deletion of HAC1 or...
IRE1 is impossible due to their synthetic lethality. However, the presented workaround is complex and unsatisfying. Would it be possible to chemically induce LBS, e.g. by inositol depletion, and use those cells (with and without full-length or ΔLD Ire1) as additional controls? It is of concern that many, if not most, of the identified genes in the Δopi3 cells have little to do with Ire1 or the UPR and are rather hallmarks of PC deficiency.

We agree with the reviewer that Fig. 6 was insufficient to support our hypothesis. As we have shown that \textit{PBI2, PIR3 and PUT1} genes are upregulated in a \textit{HAC1}-dependent manner during LBS by ChIP, we further asses the transcriptome levels of these genes during acute ER stress. As Reviewer #2 raised similar concerned, we invite Reviewer #3 to see our response to Reviewer #2, comment #1.

4. The manuscript would significantly benefit from careful copy editing. The manuscript contains numerous typos, along with awkward or grammatically incorrect sentences, that distract the reader and make the authors’ intended messages hard to grasp.

We thank the reviewer for pointing this out. We have gone through multiple rounds of corrections and we feel that the manuscript is fit for publication.
April 1, 2020

RE: JCB Manuscript #201909165R

Dr. Guillaume Thibault
Nanyang Technological University
School of Biological Sciences
60 Nanyang Drive, room 03n-28
Singapore Singapore

Dear Dr. Thibault:

Thank you for submitting your revised manuscript entitled "Stress sensor Ire1 deploys a divergent transcriptional program in response to lipid bilayer stress". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). However, as you will see from the comments from reviewer #2, there remains a concern that that differences in transcriptional programs could be due to indirect effects, which must be thoroughly addressed in a balanced manner in your final text prior to publication.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) * Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.*

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so,
5) Abstract and title: The abstract should be no longer than 160 words and should communicate the significance of the paper for a general audience. The title should be less than 100 characters including spaces.

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. ** You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies. Please also indicate the acquisition and quantification methods for immunoblotting/western blots.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
   a. Make and model of microscope
   b. Type, magnification, and numerical aperture of the objective lenses
   c. Temperature
   d. Imaging medium
   e. Fluorochromes
   f. Camera make and model
   g. Acquisition software
   h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstructions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) * Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental display items (figures and tables). At the moment, you currently have 9 such items (6 figures and 3 table). We can give you more room to accommodate the tables, however you can only have 5 SI data figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.*

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. (Please edit based on the comments of reviewer #2).

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."
13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

**It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.**

**The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.**

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Jodi Nunnari, Ph.D.
Editor-in-Chief
Journal of Cell Biology
Reviewer #1 (Comments to the Authors (Required)):

I remain puzzled by the significantly reduced levels of both HAC1U and HAC1S RNAs in ire1Δopi3Δ expressing IRE1ΔLD shown in both Figures 3E and 4A (2nd lane from the right) in the absence of Choline. The authors have not provided explanation. However, while these are interesting findings, they do not impact the overall conclusions of the revised manuscript and are something for future follow-up. Taken together, I believe that the authors have adequately addressed my concerns from the original submission and appropriate changes have been made in the revised manuscript.

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed some of the most critical concerns of the reviewers, which has improved the manuscript.

However, a few points (e.g. point 3A and 3B) critical to an appropriate, balanced discussion of the presented data have not been addressed. I can only recommend this manuscript for publications if the authors have addressed the following critical points.

1) Sensing switch in Ire1
   In the summary, the authors highlight that they identified pathways that 'activate the UPR by lipid bilayer stress through a sensing switch in Ire1'. There is no evidence in the manuscript of a sensing switch in Ire1.

   In fact, the authors stated in the response to the reviewers (point 3C): 'We agree with the reviewer that we have no experimental evidence validating our hypothesis that R537 contributes to Ire1 conformational change by LBS. Accordingly, we remove the sentence "The arginine residue located at the interface of the amphipathic and transmembrane helices of Ire1 is essential for LBS sensing, suggesting a conformational change inducing Ire1 activation (Fig. 5)" in the discussion (line 375).'

   Why does the 'switch' appear in the summary?

2) What is relevant? Ire1, Hac1 or both?
   In their response to point 3A, the authors refer to a number of examples, where the Hac1 mRNA and the protein level are regulated independently of Ire1. However, this important discussion is missing entirely in the revised manuscript. This should be corrected.
3) 4-BPA is not a chemical chaperone!
In the revised manuscript, the authors use 4-phenylbutyrate as a chemical chaperone. It was recently shown that it acts very specifically on COPII protein sorting (https://elifesciences.org/articles/26624) by competing with the binding of p24 proteins to COPII. Naturally, COPII-mediated traffic has a tremendous impact on protein and lipid transport from the ER and thus on the UPR. Either the relevant section (lines 218-225) should be removed or discussed in the light of this eLife publication on the mechanism of 4-BPA.
April 3rd, 2020

Jodi Nunnari, PhD
Editor-in-Chief
Journal of Cell Biology

Dear Prof. Jodi Nunnari,

Please find enclosed our revised manuscript entitled "Stress sensor Ire1 deploys a divergent transcriptional program in response to lipid bilayer stress" (JCB #201909165R).

We are grateful for your efforts and those of the reviewers in evaluating our revised submission. We incorporated minor changes to the manuscript to address reviewers’ comments. We also made changes to be in line with JCB formatting guidelines.

Below, we outline our responses and actions, point-by-point, to reviewer comments. With those changes, we believe that the manuscript is ready for publication in the Journal of Cell Biology.

We thank you for your efforts.

Asst Prof Guillaume THIBAULT
School of Biological Sciences, Nanyang Technological University
Adjunct Principal Investigator, Institute of Molecular and Cell Biology, A*STAR
T (65) 6592-1787 thibault@ntu.edu.sg www.thibaultlab.com
REMARKS TO AUTHORS

Please note that most changes are highlighted in red throughout the revised manuscript.

Reviewer #1 comments

1. I remain puzzled by the significantly reduced levels of both HAC1U and HAC1S RNAs in ire1Δopi3Δ expressing IRE1ΔLD shown in both Figures 3E and 4A (2nd lane from the right) in the absence of Choline. The authors have not provided explanation. However, while these are interesting findings, they do not impact the overall conclusions of the revised manuscript and are something for future follow-up. Taken together, I believe that the authors have adequately addressed my concerns from the original submission and appropriate changes have been made in the revised manuscript.

We agree with the reviewer that it is an intriguing result. At this point, we do not have evidence to provide a credible hypothesis. However, we are following up on the finding of this manuscript with experiments and we will surely investigate this point of low HAC1U and HAC1S mRNAs in ire1Δopi3Δ expressing IRE1ΔLD.

Reviewer #2 comments

1. Sensing switch in Ire1

In the summary, the authors highlight that they identified pathways that ‘activate the UPR by lipid bilayer stress through a sensing switch in Ire1’. There is no evidence in the manuscript of a sensing switch in Ire1.

In fact, the authors stated in the response to the reviewers (point 3C): 'We agree with the reviewer that we have no experimental evidence validating our hypothesis that R537 contributes to Ire1 conformational change by LBS. Accordingly, we remove the sentence “The arginine residue located at the interface of the amphipathic and transmembrane helices of Ire1 is essential for LBS sensing, suggesting a conformational change inducing Ire1 activation (Fig. 5)” in the discussion (line 375).'

Why does the ‘switch’ appear in the summary?

We agree with the reviewer that our use of “switch” was inappropriate and inaccurate. Accordingly, we replaced “switch” to “sensor” in the summary and abstract.

Updated manuscript (lines 2-3):

“Ho et al. identified pathways, beyond lipid metabolism, that are required to maintain ER integrity and, when disrupted, activate the UPR by lipid bilayer stress through a sensor in Ire1.”

Updated manuscript (lines 11-13):

“To systematically validate yeast mutants that disrupt ER membrane homeostasis, we identified a lipid bilayer stress (LBS) sensor in the UPR transducer protein Ire1, located at the interface of the amphipathic and transmembrane helices.”

2. What is relevant? Ire1, Hac1 or both?

In their response to point 3A, the authors refer to a number of examples, where the Hac1 mRNA and the protein level are regulated independently of Ire1. However, this important discussion is missing entirely in the revised manuscript. This should be corrected.

We have included the response to point 3A in the manuscript by including a new paragraph to the discussion.

Updated manuscript (lines 410-424):

“The Hac1-mediated response is assumed to be linear until ER homeostasis is restored. However, there are several lines of evidence suggesting the contrary in yeast and mammals, particularly during LBS. Previously, we demonstrated that the UPR transcriptional program is modulated through differential target gene expression depending on the source of stress in yeast and C. elegans (Koh et al., 2018; Thibault et
al., 2011). Similarly, in mammalian cells, the ATF6-modulated UPR program diverged upon LBS in comparison to proteotoxic stress (Tam et al., 2018). In agreement with these findings, the HAC1 mRNA level is enhanced by a bipartite signal, misfolded proteins and either inositol depletion or temperature shift (Leber et al., 2004). Another group demonstrated the autoregulation of HAC1 during periods of extreme and prolonged ER stress by a positive feedback loop of Hac1 binding to its promoter (Ogawa and Mori, 2004). Additionally, Gcn4, a transcriptional activator of amino acid biosynthetic genes, works synergistically with Hac1 at the promoter of UPR target genes (Patil et al., 2004). In accordance with these findings, we identified and validated PBI2 and PIR3 as genes that are upregulated only during UPRLBS, which supports a mechanism of integration of multiple stimuli to mount a divergent transcriptional response by Hac1. Additional, unidentified trans-acting factors might regulate Hac1 transcriptional factor and should be explored in future studies.”

3. 4-BPA is not a chemical chaperone!
In the revised manuscript, the authors use 4-phenylbutyrate as a chemical chaperone. It was recently shown that it acts very specifically on COPII protein sorting (https://elifesciences.org/articles/26624) by competing with the binding of p24 proteins to COPII. Naturally, COPII-mediated traffic has a tremendous impact on protein and lipid transport from the ER and thus on the UPR. Either the relevant section (lines 218-225) should be removed or discussed in the light of this eLife publication on the mechanism of 4-BPA.

We thank the reviewer to pointing this out. We have modified the result section accordingly to include Ma et al. findings.

Updated manuscript (lines 218-227):

“Next, we asked if 4-phenylbutyric acid (4-PBA) attenuates the UPR activation in opi3Δ cells. 4-PBA inhibits the aggregation of proteins and consequently suppresses the UPR (Kubota et al., 2006; Le et al., 2016; Ozcan et al., 2006; Pineau et al., 2009). More recently, 4-PBA was shown to attenuate ER retention via COPII and consequently clearing the ER of misfolded proteins (Ma et al., 2017). We monitored UPR activation using the UPRE-LacZ reporter and the HAC1 mRNA splicing assay. As expected, 4-PBA attenuated Tm-induced UPR in IRE1 cells (Fig. 3, E and F). In opi3ΔIRE1 cells, 4-PBA failed to reduce the UPR. In contrast to a previous report that 4-PBA accelerates Ire1 degradation (Mai et al., 2018), Ire1 protein levels were unchanged in IRE1 and opi3ΔIRE1 cells upon 4-PBA treatment (Fig. S3 D). These findings reinforce the notion that LBS is the main driver of the UPR in opi3Δ.”