Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly synthesised insulin receptors to the cell surface

Max Brown, Samantha Dainty, Natalie Strudwick, Adina D. Mihai, Jamie Watson, Robina Dendooven, Adrienne Paton, James Paton, and Martin Schroeder

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

RE: E18-01-0013
TITLE: Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly synthesised insulin receptors to the cell surface

Dear Dr. Shroeder:

Thank you for submitting your manuscript entitled "Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly synthesised insulin receptors to the cell surface" to Molecular Biology of the Cell. I have now received reports from two reviewers who have carefully read your manuscript and provided thorough recommendations. As you will see from the attached reports, both reviewers expressed interest in your experimental findings.

Both reviewers indicated that the manuscript would need to be revised before it could be reconsidered for publication in Molecular Biology of the Cell. The revisions include both additional experimentation and modification of the text to improve the presentation. In most cases, the additional experimental work involves repetition of the current experiments to improve image quality. Reviewer #2 suggested that the manuscript cold be trimmed by summarizing the initial experiments. I wouldn't advocate elimination of figures, as one objective of Molecular Biology of the Cell is to publish research that carefully documents the major conclusions. However, revision of the text to make the presentation more concise, yet accessible to non-specialists, is strongly recommended.

Based upon the comments of the reviewers, I encourage you to submit a carefully revised version of you manuscript that addresses the reviewers concerns. It would be most helpful if you could provide a point-by point description of the modifications that you have incorporated into the revised manuscript.

Sincerely,

Reid Gilmore
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Schroeder:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

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Reviewer #1 (Remarks to the Author):

Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly synthesised insulin receptors to the cell surface
Brown M et al (Schroder M corresponding)

This manuscript addresses the mechanisms by which the unfolded protein response (UPR) induced by endoplasmic reticulum stress can confer insulin resistance. One prevailing model is that activation of JNK via induced UPR sensor IRE1 can lead to serine phosphorylation of IRS1, which can serve as a negative feedback inhibitor of the insulin signaling pathway. Using a variety of cultured cell lines, the manuscript suggests that pharmacological induction of ER stress does not affect insulin signaling during periods up to 12 hours. There are some concerns with this line of investigation with questions about the amounts of insulin used in the treatment of the cell liness, some differences among the cell lines, and some questions about some controls. Longer periods of ER stress do appear to adversely affect insulin signaling in the cultured cell models. JNK signaling does not appear to be an underlying mechanism for the inhibition with prolonger ER stress, rather there is lowered insulin receptor trafficking from the ER to plasma membrane. Lowered amounts of mature receptor would then be available for signaling. The conclusion for the second half of the manuscript is solid at least for cell culture.
Overall, the manuscript addresses some interesting questions concerning the mechanisms by which ER stress and contribute to insulin resistance. The manuscript addresses mechanistic

questions and conveys a command for the critical steps in the UPR and experimental approaches for ER stress and. There is enthusiasm for the key findings of the manuscript. The manuscript is generally clearly written and the key conclusion about ER stress adversely affecting insulin trafficking is solid. The major reviewer concern for the manuscript involves the details of the cell culture model. There are multiple cell lines, and there are some variations in the results between them. Sometimes a result is shown for one cell line and the underlying mechanism is compared to the analysis from another cell line without all critical controls (timing, signaling events). There is also a concern about the insulin be dosed at 100 nM, and a few experiments using a more physiological concentration of 10 nM. Finally, there are no in vivo comparisons, which is some concern but understandable with the extensive number of experiments presented for the in vitro analysis.

Reviewer concerns:
1. Abstract: The abstract does not clearly set up the key questions being addressed in the manuscript. The abstract and manuscript assume that the phosphorylation sites key to the signaling pathway are already known by the reader and does not sufficiently delineate early and prolonged signaling. How does one divide early and prolonged and how would this relate to insulin resistance and diabetes? Furthermore, the abstract reads too much like a list of experimental observations and does not walk the reader through the key concepts being addressed.

2. Introduction: The first part of the introduction is effective at presenting the UPR. The larger trafficking ideas for insulin signaling need to be included here. Furthermore, the early and prolonged events need to be explained more fully, along with the roles of Akt and IRS1 signaling and protein phosphorylation.

3. Figures 1-3: Western blot panels shown here and later should include MW markers, and panels should not be too closely cropped and should be of appropriate exposure (e.g. Figs 2A-C and Fig 3A bottom panel are arguably overexposed and problematic). Fig. 1 uses 100 nM insulin. Justify this dose for the experiments. In some panels in figure S2, a more physiological dose of 10 nM is used. However, the 10 nM dose only covers early time points. Does ER stress deter insulin signaling up to 8 hours treatment with 10 nM insulin? Of importance, it is not clear whether JNK is actually activated for these ER stress conditions. JNK activation should be measured. For example p-JNK is only detectable at 10 microg/ml of Tm at 15 min in Fig. 2D. Fig. 2C appears to have an error with lane 2 showing more pT for ISR1, but 0.14 ratio compared to no insulin. Overall, the listed quantitation for Fig. 2C are problematic. It would be useful to include the cell line name in the different panels as one is moving through several different lines with similar labels.

4. The text could be streamlined a bit, and it is helpful to have paragraph breaks. Briefly walk the key insulin signaling events being measured and the rationale for the timing of the experiments. Explain the ideas behind the IRS1 feedback phosphorylation in the paragraph beginning at line 182.

5. Figs. 4-5: Line 267 describes the possible underlying mechanisms for lowered insulin receptor levels. These are not mutually exclusive and could involve one or more of these processes. Is it clear that ERAD does not participate? It is stated based on a [35S] pulse at 24 hours that lowered translation does not participate. The measurements in Fig. 5E are tough to decipher and it is this only panel with a MW marker. Furthermore, the western blots in Fig. 4A are steady state measurements at 12, 18, and 24 hours. With the GADD34 feedback system involved in translational control in the UPR and the absence of eIF2 phosphorylation measurements, it is problematic eliminating lowered insulin receptor synthesis as a contributor.

6. Figs. 6-8: These figures introduce additional cell lines HEK293 and MEF cells. Unfortunately these

cells were not tested for the early ER stress/insulin signaling features described for the other cell lines. This arguably adds a layer of uncertainty to the analysis. HEK 293 do transfect with high efficiency, and HepG2 cells can clump, but there were other cells used and one is using a GFP visualization so transfection efficiency is not paramount. Dito for the MEF cells. The dimer insulin receptor approach was clever and provided more confidence to the underlying theme of the manuscript.

Reviewer #2 (Remarks to the Author):

Previous in vivo and in vitro studies have shown that ER stress causes insulin resistance. In this manuscript, the authors examine the molecular basis for ER stress induced insulin resistance. The main findings are that reduced insulin signalling correlates with reduced levels of the mature insulin receptor in cells, whilst activation of JNK or TRB3 (previously suggested to promote insulin resistance in ER stressed cells) does not lead to inhibition of insulin signalling. Evidence is provided that ER stress inhibits maturation of the insulin receptor, leading to reduced expression at the plasma membrane. They present a model whereby long term ER stress interferes with folding and post-translational processing of the insulin receptor in the secretory pathway, causing it to be retained intracellularly. Importantly they show that restoration of insulin receptor activity, using an ingenious chimera composed of the insulin receptor cytoplasmic domain plus a membrane targeting signal which can be activated by a small molecule ligand, restores downstream insulin signalling outputs in ER stressed cells. Overall, the experiments are well designed and the data of high quality and accurately interpreted. The results are presented in a logical order and the manuscript is well written. However, it is perhaps rather too long and I wonder whether the initial experiments looking at the effect of short-term ER stress induction could be significantly summarised. The discussion is also very long and contains some repetition, so could be summarised. It would also help to have a schematic diagram to illustrate the proposed model. This would improve overall readability of the manuscript.
Specific comments to be addressed:
One prediction of the model is that the insulin receptor synthesised in ER stressed cells misfolds and is degraded by ERAD. Have the authors tested this?
It is important to note that since tunicamycin inhibits N-glycosylation, it is likely that lack of proper glycosylation (which is known to be important for correct folding) underlies effect of tunicamycin on insulin receptor levels. Thus it is critical that comparable results were observed with other ER stressors. The authors should offer a potential explanation for why insulin receptor trafficking is inhibited in ER stressed cells (eg. reduced availability of chaperones).
Explain rational for choice of cell lines.
The level of cell death observed with prolonged ER stress is a concern - how robust are conclusions based on cell populations with low viability?
MW markers should be shown on blots/gels.
Bar charts in many Figs (eg Fig 1) are too small to be easily legible.
Fig 1D if different (parts of) gels have been spliced together (this may be a pdf issue), this should be indicated with white line.
Fig 2 - quantification should be shown - the blot shown are not particularly convincing due to unequal loading between lanes.
Fig 3A - what is explanation for apparent reduction in IRS phosphorylation upon Tg treatment?
Fig 3B - is the apparent increase in IRS phosphorylation caused by loss of total IRS? Is there actually an increase in the ELISA signal? Ie how accurate is the quantification here?
Fig 3C & D - why is the level of IRS phosphorylation different in untreated HepG2 cells?

Fig 5E is not convincing and should be removed or an alternative gel provided. Longer pulse times would give better labelling of InsR.

Responses to reviewers' comments MBC-E18-01-0013

Reviewer #1

We thank the reviewer for her/his comments on our manuscript. We have improved our manuscript along the lines suggested by the reviewer. The changes in response to the reviewer's recommendations are described in detail below.

1. **Abstract: The abstract does not clearly set up the key questions being addressed in the manuscript. The abstract and manuscript assume that the phosphorylation sites key to the signaling pathway are already known by the reader and does not sufficiently delineate early and prolonged signaling. How does one divide early and prolonged and how would this relate to insulin resistance and diabetes? Furthermore, the abstract reads too much like a list of experimental observations and does not walk the reader through the key concepts being addressed.**

   Response: The abstract has been revised and focussed on presenting the key question and the key findings of our work. References to specific phosphorylation sites have been omitted from the abstract to improve its readability.

   References to 'early' and 'prolonged' ER stress signalling have been removed from the abstract for the same reason. References to 'early' and 'prolonged' ER stress signalling also have been removed from the main sections of the manuscript and replaced with timings to avoid ambiguities in the interpretation of the terminology of 'early' and 'prolonged' ER stress signalling.

   Paragraphs five and six (lines 454 – 504 in the original manuscript) have been rewritten to explain the relevance of ER stress lasting for several half-lives of the insulin receptor at the plasma membrane for insulin resistance and diabetes in more detail (lines 645 – 731).

2. **Introduction: The first part of the introduction is effective at presenting the UPR. The larger trafficking ideas for insulin signaling need to be included here. Furthermore, the early and prolonged events need to be explained more fully, along with the roles of Akt and IRS1 signaling and protein phosphorylation.**

   Response: We have added an introductory paragraph to the introduction that briefly describes the main functions of the endoplasmic reticulum in the synthesis of secretory and plasma membrane proteins to provide the overarching trafficking ideas that are relevant to insulin signalling (lines 42 – 54).

   We have added an additional paragraph to the introduction to provide background information on insulin signalling and to introduce the different proteins and phosphorylation sites that were examined in our study (lines 80 - 98).

   As described in our response to point 1, we have replaced the terminology of 'early' and 'prolonged' ER stress signalling throughout the manuscript.

3. **Figures 1-3: Western blot panels shown here and later should include MW markers, and panels should not be too closely cropped and should be of appropriate exposure (e.g. Figs 2A-C and Fig 3A bottom panel are arguably overexposed and problematic). Fig. 1 uses 100 nM insulin. Justify this dose for the experiments. In some panels in figure S2, a more physiological dose of 10 nM is used. However, the 10 nM dose only covers early time points. Does ER stress deter insulin signaling up to 8 hours treatment with 10 nM insulin? Of importance, it is not clear whether JNK is actually activated for these ER stress conditions. JNK activation should be measured. For example p-JNK is only detectable at 10 microg/ml of Tm at 15 min in Fig. 2D. Fig. 2C appears to have an error with lane 2 showing more pT**

for ISR1, but 0.14 ratio compared to no insulin. Overall, the listed quantitation for Fig. 2C are problematic. It would be useful to include the cell line name in the different panels as one is moving through several different lines with similar labels.

Response: We have added the migration positions of the nearest molecular weight standards to all Western blots. We have reexamined all Western blotting images and have removed excessive cropping of these images throughout the revised manuscript. Figures 2A-C and 3A of the original version have been removed from the revised manuscript (see paragraph 6 below).

We have added a justification for use of 100 nM insulin to the revised manuscript (lines 137 – 139). We have repeated the time course lasting up to 8 h and stimulated C_2C_{12} myotubes with 10 nM insulin for 15 min. The data from these experiments are summarised in Figure 2 of the revised manuscript. We have also used stimulation with both 10 and 100 nM insulin in all additional experiments that were added to the revised manuscript (Figures 3, 4, 11, S2, and S3 of the revised manuscript).

For early time points, i.e. ER stress lasting for ≤ 8 h, we have previously reported on JNK activation (Brown et al., 2016). This work showed that JNK is activated as early as 10 min after induction of ER stress in C_2C_{12} myotubes and 3T3-F442A cells, and as early as 30 min after induction of ER stress in Hep G2 cells. We have provided this information in the revised manuscript (lines 168 – 169) and in the original version of our manuscript (lines 127 – 128).

We now also have measured JNK activation by Western blotting for phosphorylation in its T-loop with an anti-phospho-T183-phospho-Y185-JNK antibody at time points in ER-stressed C_2C_{12} and Hep G2 cells at which we observed decreased AKT phosphorylation, i.e. 12 – 24 h of ER stress in C_2C_{12} myotubes and 18 – 36 h of ER stress in Hep G2 cells. These experiments only detected JNK activation in Hep G2 cells exposed to various thapsigargin concentrations for 18 – 36 h. The results of these experiments are described in the Results section of the revised manuscript under the title "Pharmacologic inhibition of JNKs does not rescue insulin-stimulated S473 phosphorylation of AKT in ER-stressed cells" (lines 512 – 533) and Figure 11 of the revised manuscript. Because these experiments revealed JNK activation in Hep G2 cells treated with thapsigargin, we also have investigated whether pharmacologic inhibition of JNKs with two JNK inhibitors restores insulin sensitivity. The results of these experiments are summarised in the same section of the Results chapter (lines 534 – 561 and Figure 11 of the revised manuscript). These experiments show that pharmacologic inhibition of JNKs in Hep G2 cells does not reverse the inhibitory effect of thapsigargin on insulin-stimulated S473 phosphorylation of AKT.

Figure 2 has been replaced by new data to resolve the inconsistencies in the intensities of the signals for IRS1. These new data include analysis of the effects of thapsigargin- and tunicamycin-induced ER stress on tyrosine phosphorylation of IRS1 at four specific tyrosine phosphorylation sites (Y612, Y632, Y896, and Y941 in human IRS1; Y608, Y628, Y891, and Y935 in murine IRS1, which correspond to the tyrosine phosphorylation sites in human IRS1) in response to stimulation with 10 or 100 nM insulin in C_2C_{12} myotubes (Figure 3), 3T3-F442A (Figure S2) and Hep G2 (Figure S3) cells. In addition, we have repeated the characterisation of total tyrosine phosphorylation of IRS1 in the three cell lines by immunoblotting IRS1 immunoprecipitates with an anti-phosphotyrosine antibody (Figure 4). We have improved the extraction procedure to consistently extract full-length IRS1 for the cell lines used in this work and have improved the immunoprecipitation protocol for IRS1. Because these improvements require processing of lysates onto gels or into immunoprecipitations on the same day they are prepared, we have streamlined the timings of the original experiment (Figure 2 in the original version), but have preserved these timings as much as possible. These changes include a shorter period of insulin stimulation of 5 min, instead of 15 min, which preliminary experiments showed does not affect

the results. Details of the revised methodology are given in the Materials and Methods section of the revised manuscript.

To be consistent with the methodology for extraction of IRS1 we have replaced Figure 3 of the original manuscript with new data. The ELISA data summarised in Figure 3 of the original manuscript has been replaced by Western blotting data (Figure 5 of the revised manuscript).

4. **The text could be streamlined a bit, and it is helpful to have paragraph breaks. Briefly walk the key insulin signaling events being measured and the rationale for the timing of the experiments. Explain the ideas behind the IRS1 feedback phosphorylation in the paragraph beginning at line 182.**

   Response: We have added additional paragraph breaks to the revised manuscript and have broken the Results section down into additional chapters. We have introduced the chapters
   - "ER stress does not elicit serine 307/312 phosphorylation of IRS1",
   - "ER stress for >12 h inhibits insulin-stimulated AKT phosphorylation",
   - "Decreased insulin-stimulated AKT phosphorylation correlates with depletion of the β chain of the mature insulin receptor in ER-stressed cells",
   - "Inhibition of protein synthesis and synthesis of α-β proreceptors cannot fully explain decreased insulin-stimulated S473 phosphorylation of AKT in ER stress lasting for 24 h",
   - "Unprocessed α-β proreceptors accumulate in the ER of ER-stressed cells",
   - "Genetic ablation of JNK1 and JNK2 does not protect mouse embryonic fibroblasts (MEFs) from inhibition of insulin-stimulated AKT phosphorylation by ER stress", and
   - "siRNA-mediated silencing of expression of TRB does not protect from inhibition of insulin-stimulated AKT phosphorylation by ER stress"

   to break down the text in the Results section into smaller pieces.

   We have streamlined the text in several places. A version of the revised manuscript in which all changes are tracked is attached.

   The sentences starting at line 182 in the original manuscript have been revised to "JNK inhibits tyrosine phosphorylation of IRS1 by the activated insulin receptor by phosphorylating IRS1 at S307/312. Unaltered tyrosine phosphorylation of IRS1 in ER-stressed cells (Figures 3, 4, S2, and S3) suggested that JNK, despite being activated by ER stress, does not phosphorylate IRS1 at S307/312 or that phosphorylation of IRS1 at S307/312 by JNK does not inhibit tyrosine phosphorylation of IRS1 by the insulin receptor in ER stressed cells. To distinguish between these possibilities, … " (lines 241 - 247 in the revised manuscript) to make the motivation for these experiments clearer.

5. **Figs. 4-5: Line 267 describes the possible underlying mechanisms for lowered insulin receptor levels. These are not mutually exclusive and could involve one or more of these processes. Is it clear that ERAD does not participate? It is stated based on a [35S] pulse at 24 hours that lowered translation does not participate. The measurements in Fig. 5E are tough to decipher and it is this only panel with a MW marker. Furthermore, the western blots in Fig. 4A are steady state measurements at 12, 18, and 24 hours. With the GADD34 feedback system involved in translational control in the UPR and the absence of eIF2 phosphorylation measurements, it is problematic eliminating lowered insulin receptor synthesis as a contributor.**

   Response: We agree that these processes are not mutually exclusive. To make this clearer in the revised manuscript, we have replaced the sentence starting with "To distinguish between these possibilities, …" (line 273 in the original manuscript) with the sentence "Therefore, we decided to

determine which of these processes contribute to lower levels of mature insulin receptors in ER-stressed cells" (lines 340 – 341 in the revised manuscript).

We also agree that degradation may contribute and have considered and characterised this possibility in the revised manuscript by measuring the turn-over of surface-exposed insulin receptors (Results section "ER stress does not increase the rate of insulin receptor turnover at the cell surface", lines 404 – 446 and Figure 8 in the revised manuscript).

Figure 5E has been replaced by new data, Figure 7G, in the revised manuscript. We have improved the methodology to obtain stronger signals for the [^{35}S]-methione-labelled insulin proreceptor. This included extending the labelling period to 1 h, including 2% (v/v) dialysed FBS in the labelling medium, using storage phosphor imaging instead of film exposure to detect ^{35}S-labelled proteins, and increasing the amount of [^{35}S]-methionine to label the proreceptor. We have verified that within an 1 h label, proreceptors are not significantly processed to mature receptors.

To be consistent with the experimental methodology we have also repeated the experiments to determine total protein synthesis rates, so that these are also measured in the presence of 2% (v/v) dialysed FBS. We have replaced the data for total protein synthesis rates in the original manuscript, which were obtained in the absence of serum, with the new data recorded in the presence of 2% (v/v) dialysed FBS (Figure 7B-E of the revised manuscript).

We have determined phosphorylation of eIF2α at S51 by Western blotting in cells exposed to 0.1 μM thapsigargin or 0.1 μg/ml tunicamycin for 24 h (Figure 7F and lines 362 – 369 in the revised manuscript). These experiments do not reveal any elevation of S51 phosphorylation of eIF2α at the 24 h time point. 24 h of treatment with 0.1 μM thapsigargin decreases protein synthesis rates in C_2C_{12} and Hep G2 cells, while 24 h of treatment of 3T3-F442A cells with 0.1 μM thapsigargin or 0.1 μg/ml tunicamycin increases protein synthesis rates ~2 fold. This suggests that other mechanisms contribute to control of protein synthesis rates than phosphorylation of eIF2α at S51.

6. **Figs. 6-8: These figures introduce additional cell lines HEK293 and MEF cells. Unfortunately these cells were not tested for the early ER stress/insulin signaling features described for the other cell lines. This arguably adds a layer of uncertainty to the analysis. HEK 293 do transfect with high efficiency, and HepG2 cells can clump, but there were other cells used and one is using a GFP visualization so transfection efficiency is not paramount. Dito for the MEF cells. The dimer insulin receptor approach was clever and provided more confidence to the underlying theme of the manuscript.**

Response: We have used the HEK 293 for the microscopy experiments because they adhered better to the culture dishes than the other cell lines when ER-stressed. We solely have used these cells to characterise effects of ER stress on the subcellular localisation of the insulin receptor. We have validated that ER stress decreases levels of β chains of the mature insulin receptor in HEK 293 cells (Figure 6E in the original version, Figure 9E in the revised version). These experiments showed that both tunicamycin and SubAB deplete β chains to ~60% of the level of untreated HEK 293 cells in HEK 293 cells exposed for 18 h to these drugs. Therefore, and with regard to depletion of insulin receptors, HEK 293 cells behave very similar to the cell lines mainly used in this work.

We have used the MEFs, because this allowed us to compare wild type and JNK deficient cells. In the revised manuscript we have included experiments in which we pharmacologically inhibited JNKs in Hep G2 exposed to ER stressors for 36 h (Figure 11 and lines 534 – 561 in the revised manuscript). The results from these experiments support the conclusions obtained with the MEFs, (*a*) that inhibition (or the absence) of JNKs does not restore insulin sensitivity, and (*b*) that inhibition (or the absence) of JNKs does not restore processing of insulin proreceptors.

Reviewer #2

We thank the reviewer for her/his comments on our manuscript. We have improved our manuscript along the lines suggested by the reviewer. The changes in response to the reviewer's recommendations are described in detail below.

General comments

As suggested by the reviewer, we have added a schematic to the manuscript that summarises the key findings (Figure 14, A in the revised manuscript).

The reviewer has suggested to make the manuscript shorter by summarising some of the initial experiments looking at the effect of short-term ER stress induction. We have decided against this because the monitoring editor advised against this.

The reviewer has also suggested to make the discussion shorter. We have removed the 2nd paragraph of the Discussion (lines 409 – 423 in the original manuscript), because the topic discussed in this paragraph is not of central importance to this work. We have also removed the 3rd and 4th paragraphs of the Discussion (lines 424 – 453 in the original manuscript), because the information in these paragraphs, as indicated by the reviewer, is largely repetitive to the Results section. We also have made the introduction to the 6th paragraph of the Discussion more concise (lines 472 – 476 in the original manuscript). We have revised the first paragraph of the Discussion to provide a complete summary of the main findings. We have added one paragraph to the Discussion to discuss the underlying effects that may interfere with receptor trafficking and to highlight that similar observations were made with other ER stressors than tunicamycin, which do not interfere with receptor trafficking by inhibiting N-glycosylation of the receptor. We have also revised Paragraphs five and six (lines 454 – 504 in the original manuscript) to explain the relevance of ER stress lasting for several half-lives of the insulin receptor at the plasma membrane for insulin resistance and diabetes in more detail (lines 645 – 731) as requested by reviewer #1.

Specific comments to be addressed:

1. **One prediction of the model is that the insulin receptor synthesised in ER stressed cells misfolds and is degraded by ERAD. Have the authors tested this?**

   Response: We agree with the reviewer that this is a possibility. We have, however, not yet tested this hypothesis because we believe this is part of another investigation. In addition, optimisation of [^{35}S]-methionine labelling of insulin receptors and immunoprecipitation of insulin receptors took considerable effort during the revisions (the transmembrane protein tends to aggregate in the immunoprecipitation), but we are now in a position to perform these experiments if considered essential for this work. We have, however, referred in the Discussion to the possibility that misfolded proreceptors may be degraded by ERAD (line 637 and lines 699 - 700 in the revised manuscript).

2. **It is important to note that since tunicamycin inhibits N-glycosylation, it is likely that lack of proper glycosylation (which is known to be important for correct folding) underlies effect of tunicamycin on insulin receptor levels. Thus it is critical that comparable results were observed with other ER stressors. The authors should offer a potential explanation for why insulin receptor trafficking is inhibited in ER stressed cells (eg. reduced availability of chaperones).**

   Response: We have discussed this point in the discussion (lines 618 – 644 in the revised manuscript).

3. **Explain rational for choice of cell lines.**

Response: We have used 3T3-F442A adipocytes, C_2C_{12} myotubes and Hep G2 hepatoma cells in this investigation because these cells are cell culture models of the main peripheral tissues/organs involved in glucose homeostasis. This information has been included in the revised manuscript (lines 128 – 132).

4. **The level of cell death observed with prolonged ER stress is a concern - how robust are conclusions based on cell populations with low viability?**

   Response: We have repeated the MTT tests and have normalised the activity of mitochondrial redox chains measured in these assays by reduction of MTT to cell numbers determined by crystal violet staining. This normalisation is easier, faster, and more reproducible than normalisation to protein. The results (Figure S4 in the revised manuscript) show that there is some loss of cells when cells are exposed to ER stressors for long times, but that the cells that remain in the culture dishes is not affected by ER stress.

5. **MW markers should be shown on blots/gels.**

   Response: MW markers are shown on all blots/gels in the revised manuscript.

6. **Bar charts in many Figs (eg Fig 1) are too small to be easily legible.**

   Response: All bar charts and graphs have been revised. Font and symbol sizes have been increased. The results ('stars') from statistical significance testing are now shown in the same part of each graph to increase the visibility of these results and to better distinguish these labels from the symbols for individual data points.

7. **Fig 1D if different (parts of) gels have been spliced together (this may be a pdf issue), this should be indicated with white line.**

   Response: We have investigated this figure carefully. We believe that this is an issue with conversion of a low resolution image in Microsoft Word to a PDF. Images for publication have been produced in Inkscape and are of higher resolution. We hope that these will not show this issue. We also are not aware that we have spliced together bands from different gels.

8. **Fig 2 - quantification should be shown - the blot shown are not particularly convincing due to unequal loading between lanes.**

   Response: Figure 2 has been replaced by new data to address the issue with unequal loading of IRS1 as explained in our response to point 3 of reviewer #1. All experiments have been repeated at least three times, quantified, and data have been analysed using the statistical tests as described in the Materials and Methods section and the figure legends of the revised manuscript.

9. **Fig 3A - what is explanation for apparent reduction in IRS phosphorylation upon Tg treatment? Fig 3B - is the apparent increase in IRS phosphorylation caused by loss of total IRS? Is there actually an increase in the ELISA signal? Ie how accurate is the quantification here? Fig 3C & D - why is the level of IRS phosphorylation different in untreated HepG2 cells?**

   Response: The ELISA and Western blotting data shown in Figure 3 of the original manuscript have been replaced by new data (Figure 5 of the revised manuscript). To address unequal loading of IRS1 in the immunoprecipitation experiments (Figure 2 of the original version), we have improved the extraction and immunoprecipitation protocol for IRS1. The improved extraction method for IRS1 allowed us to detect S307/312 phosphorylation of IRS1 by Western blotting (see control lanes with the anisomycin treatment in Figure 5 in the revised manuscript). The experiments have been repeated three times with 3T3-F442A and C_2C_{12} cells, and six times with

Hep G2 cells, quantified and analysed using the statistical tests described in the Materials and Methods section and the figure legends of the revised manuscript.

10. **Fig 5E is not convincing and should be removed or an alternative gel provided. Longer pulse times would give better labelling of InsR.**

Response: Figure 5E has been replaced with new data (Figure 7G in the revised manuscript). To increase the intensity of the bands for the proreceptor we have extended the labelling time to 1 h, performed the labelling in the presence of 2% (v/v) dialysed FBS, used storage phosphor imaging to detect [^{35}S]-labelled proteins, and have increased the amount of [^{35}S]-methionine to label the proreceptor. With these modifications we have obtained stronger bands for the proreceptor (Figure 7G of the revised manuscript). We have verified that within an 1 h label, proreceptors are not significantly processed to mature receptors.

To be consistent with the experimental methodology we have also repeated the experiments to determine total protein synthesis rates, so that these are also measured in the presence of 2% (v/v) dialysed FBS. We have replaced the data for total protein synthesis rates in the original manuscript, which were obtained in the absence of serum, with the new data recorded in the presence of 2% (v/v) dialysed FBS (Figure 7B-E of the revised manuscript).

REFERENCES

Brown M, Strudwick N, Suwara M, Sutcliffe LK, Mihai AD, Ali AA, Watson JN, and Schröder M (2016). An initial phase of JNK activation inhibits cell death early in the endoplasmic reticulum stress response. J Cell Sci *129*, 2317-2328.

RE: Manuscript #E18-01-0013R
TITLE: "Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly synthesised insulin receptors to the cell surface"

Dear Dr. Schroeder:

Thank you for submitting the revised version of your manuscript " Endoplasmic reticulum stress causes insulin resistance by inhibiting delivery of newly synthesised insulin receptors to the cell surface."

After reading your revised manuscript and rebuttal letter, I have concluded that the new evidence that you have added to the manuscript addresses the reviewers' concerns. I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Sincerely,
James Olzmann
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Schroeder:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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