THE EMBO JOURNAL

Manuscript EMBO-2010-75710

The Unfolded Protein Response Transducer IRE1α Prevents ER Stress-Induced Hepatic Steatosis

Kezhong Zhang, Shiyu Wang, Jyoti Malhotra, Justin R. Hassler, Sung Hoon Back, Guohui Wang, Lin Chang, Wenbo Xu, Hongzhi Miao, Roberta Leonardi, Y. Eugene Chen, Suzanne Jackowski and Randal J. Kaufman

Corresponding author: Randal J. Kaufman, HHMI & University of Michigan

Review timeline:

Submission date:	18 August 2010
Editorial Decision:	29 September 2010
Revision received:	12 January 2011
Editorial Decision:	28 January 2011
Accepted:	28 January 2011

Transaction Report:

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

1st Editorial Decision 29 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. After some delay due to difficulties in finding suitable and willing referees for this manuscript during the past summer holiday period it has now finally been seen by two referees whose comments are shown below. As you will see while both referees are supportive regarding publication of the study here in principle referee 1 raises a number of major concerns that affect your conclusions regarding unchallenged mice and regarding specific aspects of the hepatic lipid metabolism that in his/her view would need to be addressed by a number of additional experiments. On balance, I have thus come to the conclusion that we should be able to consider a revised version of this manuscript in which all these concerns need to be addressed in an adequate manner and to the satisfaction of referee 1.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referee.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is an interesting study of the role of IRE1a in the maintenance of hepatic lipid homeostasis during ER stress. The authors conclude that IRE1a plays a critical role in hepatic lipid metabolism with or without ER stress. The strengths of the work include the authors expertise in studying ER stress and the UPR, the generation of a unique liver specific knockout of IRE1a, a recent surge of interest and data linking ER stress and lipid metabolism, and the broad range of studies completed. The weaknesses include the absence, in this reviewer's opinion, of a significant phenotype in the knockout mice without tunicamycin (TM) treatment, the use of only TM, a very potent cause of ER and the UPR, and the lack of expertise of the authors in hepatic lipid metabolism. Additionally, the experiments with bortezomib do not add to the central them/concept of the paper. Overall, while the authors show that the absence of hepatic IRE1a results in much greater UPR coupled with alterations in hepatic lipid metabolism leading to hepatic steatosis, they have not convinced this reviewer that IRE1a is important without a high level of ER stress.

Specific issues:

1. Why did the authors take what seems to be an unusual breeding strategy - hetero floxed/heteronull mice crossed with albumin Cre mice rather than homozygous floxed x albumin Cre? The latter would have meant that the controls were wt for IRE1α rather than controls that were hetero knockouts (Figure 1).

2. Figure 2 table 1 - The gene changes are interesting - but without TM there is really not much happening

3. Figure 3 - bottom page 7-top page 8 : GRP78 and GRP94 appear to be more than "slightly" reduced in the knockout livers - are there just single experiments? Why not repeats and stats?

4. Figure 4 data - convincing data (typo Figure 3F at this point should be 4F) but what is mechanism for the mild steatosis in the non-TM treated mice?

5. Figure 4 - page 12: the authors say that CEBP beta and delta are up in ko mice - but stats say that is significant only with TM

6. The authors state that PPAR gamma1 levels are up, especially in TM treated ko mice - but stats say that is significant only for TM. Importantly, the authors do not seem to be aware of the evidence that PPAR gamma2 is specifically involved in hepatic steatosis models; this paper needs to look at PPAR gamma2 What about classical PPAR targets like aP2 or CD36? What about FSP27? The authors need to differentiate between PPARgamma 1 and 2 in the text - the PCR seems to be for PPARgamma1 but, as noted above, they must then look at PPARgamma2 as well. Additionally, the western blot is for which form of PPARgamma - both or gamma1?

7. LXR and ChREBP are said, in the text, to be increased (without mentioning TM) - but stats say only with TM If LXR is up - why is SREBP-1c not increased. Also, it is hard to argue de novo lipogenesis is up in the liver with no increase in either SREBP-1c or FASN

8. The text says that there is increased expresssion of ADRP and FIT proteins (both?) The figures show that ADRP and FIT2 are only increased with TM and that FIT1 is not at all.

9. The data for SCD1, DGAT1, DGAT2, and ACC1 are more impressive although again, except for the DGATs, the significance is only with TM. -Additionally, are the expressions of DGATs with TM expression greater than in ko alone? Need to show stats. Did the authors look at fatty acid oxidation genes? What about lipid uptake genes? In a study like this, key experiments would be to actually measure, biochemically, de novo lipogenesis and fatty acid oxidation; it is clear from many studies that gene expression does not always correlate with actual lipogenesis or oxidation rates.

10. Figure 7 - A/B - The authors need to have the methods for these gels and westerns in the paper - it looks like apoB aggregated at the top of the gels in some instances; this is common even with 4% gels. Second, when discussing TM treated livers, it would be better to say in that the knockout mouse livers, apoB100 did not decrease as much while plasma decreased more - this would put the TM treated liver data in the context of the non-TM treated mice. As for apoB48, the gels are confusing - the liver B48 looks like it is reduced by TM in the wt mice but not affected in the knockout mice whereas in plasma, apoB48 looks increased in both sets but less so in the knockouts. In figure 7C, the gels are very unimpressive and not really informative. Additionally, prior studies from Adeli lab and Ginsberg lab showed that ER stress led to decreased apoB100 secretion with increased apoB100 degradation - these data suggest no degradation but simply no secretion - seems unlikely based on all that has been published about the regulation of apoB secretion.

11. Suppl figure 3 - not clear of the relevance of these data to the central theme of the paper. Why pick these proteins - I suggest dropping these data.

12. Figure 8 - these are interesting data but inhibiting the proteasome does so many things. What about apoB in the livers and the plasma after Bortezomib rx? Does Bortezomib cause fatty liver - this reviewer could find no evidence for such an effect in the literature or the drugs package insert.

13. Discussion: Page 19 - the evidence for a significant role of IRE1a in non-TM model is very weak

14. Page 20 - when discussing PPAR in liver, the authors again miss differentiating between PPARgamma2 vs 1

15. Page 21 - the discussion of the lipogenesis pathway is weakened by lack of role for SREBP-1c and FASN. If SCD1 and the DGATS are up, where are the fatty acids coming from

16. Page 21 bottom - the authors raise the question regarding the relationship between lipid droplet enzymes and ER stress - but they have shown that DGAT1 and DGAT2 - one or both of which are critical for droplet formation - are increased in the ko mice with or without TM

17. Page 22 - as noted above, there is no mention of other works that show reduced apoB secretion via ER stress mediated pathways but with apoB degradation

Referee #2 (Remarks to the Author):

Kaufman and colleagues have previously shown that all three arms of the UPR are important for protection of hepatocytes from lipid accumulation/steatosis during ER stress (induced by sublethal systemic tunicamycin administration). This suggests that general ER homeostasis is required to avoid hepatic steatosis, not specific targets of one of the UPR pathways. Among several genetic models, conditional Ire1alpha (albumin-Cre//Ire1alphaflox/-) mice were employed in that study. This manuscript by Xhang et al. describes these mice in exquisite detail. Analysis of liver in development and after tunicamycin challenge shows that Ire1alpha deficiency produces very mild ER impairment and lipid accumulation in untreated mice, and confirms marked lipid accumulation/steatosis after tunicamycin challenge. Comprehensive transcriptomic analysis and biochemical analysis of the three arms of UPR show that after tunicamycin treatment, a persistant, maladaptive CHOP response is activated associated with hepatocyte apoptosis. Absence of Ire1alpha derepresses transcriptional activators of lipid biosynthesis and limits lipid secretion during ER stress and some of these perturbations were confirmed in cultured cells to rule out adaptive changes due to chronic deficiency of Ire1alpha. Finally, bortezomib-induced ER stress (a

proteasome inhibitor that impairs ERAD) produced similar defects in the context of Ire1alpha deficiency.

The manuscript is clearly written, rich in data, beautifully illustrated, and appropriately interpreted, discussed and referenced.

Although the major findings of this paper initially confirm the prior study by the same group, or other single findings from other studies in other cultured celltypes with ER stress, the comprehensive analysis of ER stress-induced hepatic steatosis in the absence of Ire1alpha allows special insights about how different arms of the UPR interact dynamically in both acute and chronic ER stress, and about what molecular events accompany the transition from adaptive to maladaptive ER stress response-all in tissue (not cultured fibroblasts). As a consequence, this paper will be of broad interest to the cell biology and misfolded protein disease communities.

There are only minor suggestions:

1) the Yamamoto et al citation on page 5 is missing the year.

2) Some of the names or annotations are cropped by column lines in Table 1.

3) The significant differences between CTL and KO gene expression without TM injection might be marked with an asterisk to attract the reader's attention in Table 1.

1st Revision - authors' response 12 January 2011

Point-to-point responses to the reviewers' comments

Reviewer #1:

Major comments: *this is an interesting study of the role of IRE1a in the maintenance of hepatic lipid homeostasis during ER stress. The authors conclude that IRE1a plays a critical role in hepatic lipid metabolism with or without ER stress. The strengths of the work include the authors expertise in studying ER stress and the UPR, the generation of a unique liver specific knockout of IRE1a, a recent surge of interest and data linking ER stress and lipid metabolism, and the broad range of studies completed. The weaknesses include the absence, in this reviewer's opinion, of a significant phenotype in the knockout mice without tunicamycin (TM) treatment, the use of only TM, a very potent cause of ER and the UPR, and the lack of expertise of the authors in hepatic lipid metabolism. Additionally, the experiments with bortezomib do not add to the central them/concept of the paper. Overall, while the authors show that the absence of hepatic IRE1a results in much greater UPR coupled with alterations in hepatic lipid metabolism leading to hepatic steatosis, they have not convinced this reviewer that IRE1a is important without a high level of ER stress.*

Response: We thank the reviewer for the valuable comments. As suggested, we performed additional experiments to demonstrate the role of IRE1α in hepatic lipid metabolism by using non-TM, pathophysiologic stress models. First, we utilized an animal model in which secretion of a misfolding-prone human blood clotting factor VIII protein (hFVIII) from hepatocytes was reduced (Kaufman et al, 1997; Miao et al, 2004). Because of the accumulation of hFVIII in the ER in hepatocytes, ER stress was induced in the liver of mice expressing the misfolding-prone *hFVIII* transgene. In support of the role of IRE1α in preventing ER stress-induced hepatic steatosis, *Ire1α*-null mice displayed more severe hepatic steatosis, compared to the control mice, upon expression of the *hFVIII* transgene in the liver (Figure 8G-I). Consistently, secretion of triglycerides was decreased in plasma while the levels of hepatic triglycerides were increased in the liver tissue in the *Ire1α*-null mice after expression of the *hFVIII* transgene. Additionally, expression of key lipogenic genes, including *Dgat2*, *Acc1*, *Scd1*, *Adrp*, and *ChREBP*, was increased in the *Ire1α*-null mice upon expression of the *hFVIII* transgene (Figure 8I). Secondarily, we tested the role of IRE1α in

maintaining hepatic lipid homeostasis using an animal model of partial hepatectomy, the surgical removal of liver (Fausto et al, 2006). In the clinic, hepatectomies are performed for removal of the liver for liver transplantation or for surgical removal of tumors (carcinoma or hepatoma) from the liver (Fausto, 2001). Supporting the role of IRE1α in preventing stress-induced hepatic steatosis, the liver of *Ire1α*-null mice displayed profound steatosis after partial hepatectomy, as reflected by accumulation of hepatic lipid droplets, increased hepatic triglycerides, and decreased plasma triglycerides (Supplemental figure 7). Together, these additional non-TM pathophysiologic stress models further confirmed that the UPR transducer IRE1α plays a critical role in preventing stress-induced hepatic steatosis.

Specific issues:

1. Why did the authors take what seems to be an unusual breeding strategy - hetero floxed/heteronull mice crossed with albumin Cre mice rather than homozygous floxed x albumin Cre? The latter would have meant that the controls were wt for IRE1α rather than controls that were hetero knockouts (Figure 1).

Response: Previously we successfully generated an IRE1α heterozygous mice with an *Ire1α* null allele in which exons 7-14 of the *Ire1α* gene were deleted (Lee et al, 2002; Zhang et al, 2005). The IRE1α heterozygous mice showed no difference from the wild-type mice in the ER stress response, immune response, and lipid metabolism (Zhang et al, 2005) (data not shown). To achieve the highest efficiency of *Ire1α* deletion, we crossed heterozygous *Ire1α* floxed/deletion mice, rather than homozygous *Ire1α* floxed mice, with albumin CRE transgenic mice. Mice with an *Ire1a* floxed allele and an *Ire1a* null allele and the albumin-Cre transgene only require deletion of a single allele to generate the null phenotype. Matching this breeding strategy, we chose littermate mice bearing an *Ire1α* floxed allele, a wild-type *Ire1α* allele, and the albumin-*Cre* transgene as control mice. This type of control mice enables us to exclude the possible artifacts caused by the flox allele and *Cre* transgene. We believe that the data obtained with this breeding strategy and control system is reliable in reflecting the involvements of IRE1α in ER stress response and hepatic lipid homeostasis *in vivo*.

2. Figure 2 table 1 - The gene changes are interesting - but without TM there is really not much happening.

Response: Although *Ire1a* deletion did not significantly affect liver phenotype, upon detailed gene expression analysis, we identified significant differences in expression of numerous genes between the *Ire1α*-null and control mice in the absence of TM challenge. The difference in gene expression profiles between *Ire1α*-null and control liver (without TM challenge) can be observed in Figure 2A (upper panel). To further discern the gene expression in the absence of IRE1α under the non-stressed condition, we performed additional bioinformatics statistical analysis based on the micro-array data obtained from *Ire1α*-null and control mice without TM challenge (Supplemental figure 1A and B). The analysis revealed 64 genes whose expression was decreased and 57 genes whose expression was increased in the *Ire1α*-null liver (at least 1.5-fold change, $P<0.05$), compared to that in the control liver, without TM challenge (Supplemental figure 1 A-B).

3. Figure 3 - bottom page 7-top page 8 : GRP78 and GRP94 appear to be more than "slightly" reduced in the knockout livers - are there just single experiments? Why not repeats and stats?

Response: We thank the reviewer for pointing out this issue. Our Western blot analysis reproducibly showed that expression of GRP78 and GRP94 proteins was decreased in the *Ire1α*-null liver. The experiments were repeated at least 4 times with different experimental samples. We have revised our description in the text accordingly.

4. Figure 4 data - convincing data (typo Figure 3F at this point should be 4F) but what is mechanism for the mild steatosis in the non-TM treated mice?

Response: We indeed observed mild hepatic steatosis and decreased plasma lipids in the *Ire1α*-null mice without TM challenge (Figure 4A, E and F). Based on our gene expression analysis, expression of genes involved in the endomembrane protein secretion machinery, including Sec61α1, Sec24d, and Tmed3, was slightly decreased in the *Ire1α*-null liver under the non-stressed condition (Table 1) (Figure 2C). Additionally, we also observed slight increases in expression of lipogenic genes encoding C/EBPβ, C/EBPδ, PPARγ1, LXRα, ChREBP, ADRP, FIT1, FIT2, DGAT1 and DGAT2 in the liver of *Ire1α*-null mice under the non-TM treated condition (Figure 5A-B), in which basal level of metabolic/physiologic ER stress should be existing (Acosta-Alvear et al, 2007). All these could partially account for the mild steatosis in the *Ire1α*-null liver. In the revised manuscript, we have added a paragraph in the discussion (Page 22) regarding the possible mechanism for the mild hepatic steatosis in non-TM treated *Ire1α*-null mice.

5. Figure 4 - page 12: the authors say that CEBP beta and delta are up in ko mice - but stats say that is significant only with TM

Response: We agree with this comment that C/EBPβ and C/EBPδ were slightly up-regulated in knockout mice while this up-regulation becomes significant after TM challenge. Accordingly, we modified the statement as "Compared to livers from control $Ire1\alpha^{Hepfe/+}$ mice, the livers from $Ire1\alpha^{Hepfe/-}$ mice displayed slightly increased expression of the *C/ebpβ* and *C/ebpδ* mRNAs, and this increase became statistically significant after TM treatment (Figure 5A)."

6. The authors state that PPAR gamma1 levels are up, especially in TM treated ko mice - but stats say that is significant only for TM. Importantly, the authors do not seem to be aware of the evidence that PPAR gamma2 is specifically involved in hepatic steatosis models; this paper needs to look at PPAR gamma2 What about classical PPAR targets like aP2 or CD36? What about FSP27? The authors need to differentiate between PPARgamma 1 and 2 in the text - the PCR seems to be for PPARgamma1 but, as noted above, they must then look at PPARgamma2 as well. Additionally, the western blot is for which form of PPARgamma - both or gamma1?

Response: As pointed out by the reviewer, PPARγ has two major isoforms, γ1 and γ2, generated from the same gene by alterative splicing (Fajas et al, 1997; Vidal-Puig et al, 1997). PPARγ2 is highly expressed in adipose tissue and is upregulated in steatotic livers of ob/ob mice, while PPARγ1 is found at low levels in many tissues, including fat and liver tissues (Dubuquoy et al, 2002; Musso et al, 2009; Tontonoz et al, 1994; Yamazaki et al, 2010; Zhang et al, 2006). It was documented that enhanced expression of hepatic PPARγ2 stimulates hepatic steatosis (Schadinger et al, 2005; Yamazaki et al, 2010; Zhang et al, 2006). PPARγ1 and PPARγ2 are very similar in including DNA binding, ligand binding, and interaction with coactivators that are mediated by identical domains. It has been proposed that both PPARγ1 and PPARγ2 may contribute to formation of fatty liver, although PPARγ2 is more effective in activating the transcription of adipogenic and lipogenic genes than PPARγ1 (Gavrilova et al, 2003; Matsusue et al, 2003; Vidal-Puig et al, 1997; Yamazaki et al, 2010; Zhang et al, 2004).

We examined expression of the *Pparγ2* gene in the liver of *Ire1α*-null and control mice after TM treatment as well as in the liver and fat tissues of mice after the high-fat diet (42% fat) for 2 months. Consistent with the literature, expression of the *Pparγ2* mRNA was increased in the liver and fat tissues of mice after the high-fat diet. The real-time PCR amplification threshold ct values for *Pparγ2* with liver tissue RNA samples from high-fat-fed mice were around 28-29; and ct values in fat tissue of high-fat-fed mice were around 25 (Supplemental figure 3). In contrast, the ct values for *Pparγ2* with the liver tissue RNA from the *Ire1α*-null and control mice after TM challenge were away above 32. These results suggest that expression levels of the *Pparγ2* mRNA in the liver were extremely low in TM-induced hepatosteatosis, although hepatic *Pparγ2* was induced in obesity-induced hepatosteatosis (Supplemental figure 3). In comparison, the levels of the *Pparγ1* mRNA were slightly increased in the livers from $Ire1\alpha^{Hepfe/-}$ mice in the absence of TM challenge, and this different became statistically significant after TM injection (Figure 5A). These results suggest that PPARγ1 is the major PPARγ isoform that is involved in TM-induced hepatic steatosis.

As suggested, we examined induction of the PPARγ target gene, including *aP2*, *Fat/Cd36*, and *Fsp27*, in the liver tissues of *Ire1α*-null and control mice in response to TM challenge. Consistent with the increased hepatic steatosis, expression of all these genes was increased in the liver of the *Ire1α*-null mice after TM treatment, compared to that in the control mice (Supplemental figure 4).

Regarding the Western blot analysis for PPARγ, we used an antibody that reacts with both PPARγ1 and PPARγ2. Because PPARγ2 was not detected at the mRNA level in the liver tissue in the absence or presence of TM challenge, the Western blot results in Figure 5D likely reflected PPARγ1. In the revised manuscript, we have added the related description/discussion regarding PPARγ in the result and discussion sections (Page 12; Page 21).

7. LXR and ChREBP are said, in the text, to be increased (without mentioning TM) - but stats say only with TM. If LXR is up - why is SREBP-1c not increased. Also, it is hard to argue de novo lipogenesis is up in the liver with no increase in either SREBP-1c or FASN.

Response: We thank the reviewer for pointing this out. We have revised the related description on the induction of LXR and ChREBP in the liver of *Ire1α*-null and control mice. In the revised manuscript, we emphasize that increased expression of the *LXR* and *ChREBP* genes in the *Ire1α*-null liver became significant only after the mice were challenged with TM.

We repeated Western blot analysis for expression of SREBP-1c in the *Ire1α*-null and control liver. Previously, we used an anti-SREBP1 antibody from Santa Cruz Biotechnologies for the Western blot analysis. We realized that the old antibody mainly detected the SREBP1a isoform. Therefore, our previous Western blot result on SREBP1 may be misleading with regard to the involvement of SREBP1c in TM-associated lipogenesis. In the revised manuscript, we used a specific anti-SREBP1c antibody from Themo Scientific for the Western blot analysis with the liver tissue samples from *Ire1α*-null and control mice under TM treatment. The new results indicated that TM treatment decreased levels of SREBP1c precursor, but not its activated/mature form, in both *Ire1α*-null and control liver. Indeed, after TM treatment, the levels of mature/active SREBP1c protein were even slightly increased in the *Ire1α*- null liver, compared to that in the control liver (Figure 5D). In contrast to the observation on active SREBP-1c protein levels, levels of the mRNA encoding SREBP1c, which reflected the expression of SREBP1c precursor, were reduced by TM treatment, in both *Ire1α*-null and control liver (Supplemental figure 5). The mechanism by which ER stress differentially modulate mRNA and protein levels of SREBP1c and its targets including FASN in the liver is an interesting question to be investigated in the future. Please also see our responses to questions 9 and 15 regarding the inconsistency between gene transcription induction and lipogenesis phenotype in which feedback regulation may play an important role.

8. The text says that there is increased expression of ADRP and FIT proteins (both?) The figures show that ADRP and FIT2 are only increased with TM and that FIT1 is not at all.

Response: We thank the reviewer for pointing this out. TM did not significantly increase the *FIT1* mRNA levels, although the levels of the *FIT1* mRNA in the *Ire1α*-null liver were higher than that in the control liver after TM treatment. In comparison, levels of the *FIT2* mRNA were significantly increased in the *Ire1α*-null liver after TM treatment. We have revised the related result interpretation accordingly.

9. The data for SCD1, DGAT1, DGAT2, and ACC1 are more impressive although again, except for the DGATs, the significance is only with TM. -Additionally, are the expressions of DGATs with TM expression greater than in ko alone? Need to show stats. Did the authors look at fatty acid oxidation genes? What about lipid uptake genes? In a study like this, key experiments would be to actually measure, biochemically, de novo lipogenesis and fatty acid oxidation; it is clear from many studies that gene expression does not always correlate with actual lipogenesis or oxidation rates.

Response: We agree with this reviewer that gene expression does not necessarily correlate with lipogenesis and oxidation rates. In the revised manuscript, in addition to the lipogenic genes, we have measured induction of the genes involved in fatty acid oxidation, including *Acox1*, *Cpt1α*, and

Cyp4a10, and the genes involved in fatty acid uptake/transport, including *Fatp2*, *Fat/Cd36*, *Fabp1*, and *Fabp4(aP2)*, in the livers of *Ire1α*-null and control mice in response to TM treatment. The result showed that induction of the genes involved in fatty acid oxidation and uptake was slightly increased in the *Ire1α*-null liver after TM treatment (Supplemental figure 4). Additionally, we also measured radiolabeled acetate incorporation and did not detect any difference between the *Ire1α*-null and control primary hepatocytes (data not shown). In addition, long fasting did not detect any difference in ketone body level between the knockout and control mice. The related results are now discussed in the revised manuscript (Page 14).

10. Figure 7 - A/B - The authors need to have the methods for these gels and westerns in the paper - it looks like apoB aggregated at the top of the gels in some instances; this is common even with 4% gels. Second, when discussing TM treated livers, it would be better to say in that the knockout mouse livers, apoB100 did not decrease as much while plasma decreased more - this would put the TM treated liver data in the context of the non-TM treated mice. As for apoB48, the gels are confusing - the liver B48 looks like it is reduced by TM in the wt mice but not affected in the knockout mice whereas in plasma, apoB48 looks increased in both sets but less so in the knockouts. In figure 7C, the gels are very unimpressive and not really informative. Additionally, prior studies from Adeli lab and Ginsberg lab showed that ER stress led to decreased apoB100 secretion with increased apoB100 degradation - these data suggest no degradation but simply no secretion - seems unlikely based on all that has been published about the regulation of apoB secretion.

Response: First**,** as suggested, we provided the detailed information regarding the methods for ApoB Western blot analysis in the revised manuscript. Second, we repeated ApoB Western blot analyses with the liver tissue and plasma samples from *Ire1α*-null and control mice. In the revised version, we include new Western blot data that demonstrate ApoB100 and ApoB48 species in plasma or liver tissue of mice under non-TM treated condition (Figure 7B). Without TM challenge, both ApoB100 and ApoB48 can be detected in liver tissue and plasma of *Ire1α*-null and control mice. Compared to the control mice, levels of ApoB100 and ApoB48 were only marginally altered in the plasma of *Ire1α*-null mice under non-TM treated condition. The difference in plasma and hepatic ApoB48 levels between the *Ire1α*-null and control mice became apparent after TM treatment (Figure 7A and B). However, TM treatment significantly suppressed production of ApoB100 in the liver and plasma of both *Ire1α*-null and control mice (Figure 7 A and B). Nevertheless, less plasma ApoB100 and ApoB48 were detected in the *Ire1α*-null mice, while more hepatic ApoB100 and ApoB48 were present in the *Ire1α*- null mice, compared to the control mice, after TM treatment. This observation indicated that TM reduced the secretion of ApoB proteins and this effect was more profound in the *Ire1α*-null mice. In turn, the deteriorated ApoB secretion could lead to more severe hepatic steatosis in the *Ire1α*-null mice than the control mice under the ER stress condition. Additionally, we can observe degraded ApoB100 signals in the *Ire1α*-null and control mice under non-TM treated condition (Figure 7A and B). However, we failed to observe significant ApoB100 or its degraded species after TM treatment. We think the difference in stress-induced ApoB100 degradation between our result and reported studies may be due to the different ER stressors (Ota et al, 2008; Qiu et al, 2009; Rutledge et al, 2009). As mentioned above, TM treatment significantly suppressed the production of ApoB100. This may cause the difficulty in observing degradation of ApoB100 in the liver and plasma samples after TM treatment. As the reviewer suggested, we clarified the effects of ER stress on ApoB levels in the revised manuscript (Page 23; Pages 15-16).

In our original manuscript, we used immortalized *Ire1α*-null and control hepatocyte cell lines for pulse-chase analysis of ApoB secretion. In the revised manuscript, the pulse-chase experiments were performed using mouse primary hepatocytes, which maintain high efficiency of VLDL secretion. The experimental details and result interpretation about hepatocyte isolation and pulse-chase analysis of ApoB secretion were included in the manuscript. Based on the results of our pulse-chase experiments with primary hepatocytes, we found that without TM treatment, the secretion rates of both ApoB100 and Apo48 were comparable between null and control hepatocytes (Figure 7C-D). After 6 hours of TM treatment, we found the secretion rates of ApoB48 were decreased in a greater extent in the *Ire1α*-null hepatocytes than control hepatocytes (Figure 7C-D). This observation is consistent with more dramatic reduction of ApoB in the plasma of *Ire1α*-null mice, compared to control mice, when they were challenged with TM (Figure 7A-B). Moreover, in either IRE1α-sufficient or deficient hepatocytes, we did not detect any secreted ApoB100, which was consistent with the ApoB-Western blot analysis of the liver and plasma samples from the TM-treated *Ire1α*-

null and control mice (Figure 7A-B). In summary, according to the reviewer's suggestion, we improved ApoB-Western blot analysis and pulse-chase analysis and drew a conclusion that IRE1α is required for efficient ApoB secretion, which is critical for maintaining hepatic lipid homeostasis under ER stress condition.

11. Suppl figure 3 - not clear of the relevance of these data to the central theme of the paper. Why pick these proteins - I suggest dropping these data.

Response: We agree with the sentiment of this reviewer and have removed the data on the acute phase response protein secretion from the revised manuscript.

12. Figure 8 - these are interesting data but inhibiting the proteasome does so many things. What about apoB in the livers and the plasma after Bortezomib rx? Does Bortezomib cause fatty liver - this reviewer could find no evidence for such an effect in the literature or the drugs package insert.

Response: We agree that inhibiting proteasome by Bortezomib affects many processes. Regarding the potential involvement of Bortezomib in hepatic steatosis, published reports have suggested possible toxic effects of bortezomib on hepatitis and steatosis (Hernandez-Espinosa et al, 2008; Rosinol et al, 2005; Rutkowski et al, 2008). As a proteasome inhibitor, one major effect of Bortezomib is inhibition of 26S proteasome activity, leading to accumulation of unfolded and/or misfolded proteins in the ER, a condition referred to as ER stress (Fels et al, 2008; Fribley et al, 2004; Lee et al, 2003; Nawrocki et al, 2005). Indeed, our results confirmed that Bortizomib is a strong ER stress inducer in the liver (Figure 8C). In support of ER stress-induced hepatic steatosis, Bortezomib was found to induce mild liver steatosis in wild-type mice, as reflected by the accumulation of hepatic lipid droplets, elevated hepatic TG levels, and increased expression of lipogenic regulators, and this effect was exacerbated by the deletion of IRE1α (Figure 8A-D). Therefore, our data not only support a role for IRE1α in maintaining hepatic lipid homeostasis upon ER stress, but also confirm potential hepatoxicity of Bortezomib treatment. We believe this information should be considered by physicians and/or pharmacists when Bortezomib is used as an anti-cancer drug. We have incorporated the related information into the revised manuscript.

13. Discussion: Page 19 - the evidence for a significant role of IRE1a in non-TM model is very weak.

Response: As suggested, we performed additional experiments to demonstrate the role of IRE1α in hepatic lipid metabolism by using two non-TM stress models: (1) an animal model in which secretion of a misfolding-prone human blood clotting factor VIII protein (hFVIII) from hepatocytes was reduced (Kaufman et al, 1997; Miao et al, 2004). Because of the accumulation of FVIII in the ER of hepatocytes, ER stress was induced in the liver of mice expressing the misfolding-prone hFVIII transgene (Malhotra et al, 2008). In support of our hypothesis, *Ire1α*-null mice displayed more severe hepatic steatosis and increased expression of lipogenic genes, compared to the control mice, upon expression of hFVIII (Figure 8G-I). Consistently, hepatic triglyceride levels were increased while plasma triglyceride levels were decreased in the *Ire1α*-null mice after expression of hFVIII (Figure 8H), thus confirming the role of IRE1α in non-TM stress-induced hepatic steatosis. (2) We tested the role of IRE1α in hepatic lipid homeostasis using an animal model of partial hepatectomy, the surgical removal of the liver in liver transplantation or tumor (carcinoma or hepatoma) removal (Fausto, 2001; Fausto et al, 2006). Supporting the role of IRE1α in maintaining hepatic lipid homeostasis, the liver of *Ire1α*-null mice displayed profound steatosis after partial hepatectomy, as reflected by chronic accumulation of hepatic lipid droplets, increased liver triglyerides, and decreased plasma triglycerides (Supplemental figure 7). Together, these additional non-TM pathophysiologic stress models further confirm that IRE1α plays a critical role in preventing stress-induced hepatic steatosis. The related results have been discussed in the revised manuscript.

14. Page 20 - when discussing PPAR in liver, the authors again miss differentiating between PPARgamma2 vs 1.

Response: In our revised manuscript, we have provided additional information regarding PPARγ1 and PPARγ2 expression in the liver (Supplemental figure 3). We have also incorporated data interpretation and discussion regarding PPARγ1 and PPARγ2 in the liver after TM challenge into the revised manuscript (please also see our response to question 6).

15. Page 21 - the discussion of the lipogenesis pathway is weakened by lack of role for SREBP-1c and FASN. If SCD1 and the DGATS are up, where are the fatty acids coming from.

Response: As we described in our response to the question 7, we repeated Western blot analysis for the expression of SREBP-1c in *Ire1α*-null and control livers after TM treatment by using a specific anti-SREBP-1c antibody (from Themo Scientific). The new results indicated that TM treatment decreased levels of SREBP1c precursor, but not the activated/mature form, in both *Ire1α*-null and control livers. After TM treatment, the levels of mature/active SREBP1c protein were even slightly increased in the *Ire1α*-null livers, compared to that in the control livers (Figure 5D). This is consistent with the increased lipogenesis in the *Ire1α*-null liver after TM treatment. Interestingly, the quantitative real-time PCR analysis showed that mRNA levels encoding SREBP1c and its target *Fasn* mRNA were reduced by TM treatment (Supplemental figure 5). Apparently, TM challenge differentially modulates mRNA and protein levels of SREBP1c and its related targets in an IRE1α-dependent manner, an interesting phenomenon that deserves future investigation. Additionally, it has been known that hepatic lipogenesis is a tightly-regulated process in which feed-back regulation plays an important role in maintaining hepatic lipid homeostasis. Therefore, specific gene expression may not positively correlate with hepatic lipogenic phenotype. In the revised manuscript, we have discussed these issues (Pages 13-14).

16. Page 21 bottom - the authors raise the question regarding the relationship between lipid droplet enzymes and ER stress - but they have shown that DGAT1 and DGAT2 - one or both of which are critical for droplet formation - are increased in the ko mice with or without TM.

Response: We thank the reviewer for pointing this out. Our data show that expression of DGATs was increased in the *Ire1α*-null liver, especially after TM treatment (Figure 5A-B). This is consistent with hepatic TG accumulation in the *Ire1α*-null liver, given the roles of DGATs in TG synthesis and in lipid droplet formation. In the revised manuscript, we discussed the relationship between ER stress-induced gene expression and lipid droplet formation in the *Ire1α*-null liver.

17. Page 22 - as noted above, there is no mention of other works that show reduced ApoB secretion via ER stress mediated pathways but with ApoB degradation.

Response: As suggested, we have mentioned the findings from other groups regarding ApoB secretion and degradation through ER stress-associated pathways in the result and discussion sections (Ota et al, 2008; Qiu et al, 2009; Rutledge et al, 2009) (Page 23).

Reviewer #2:

Major comments: *Kaufman and colleagues have previously shown that all three arms of the UPR are important for protection of hepatocytes from lipid accumulation/steatosis during ER stress (induced by sublethal systemic tunicamycin administration). This suggests that general ER homeostasis is required to avoid hepatic steatosis, not specific targets of one of the UPR pathways. Among several genetic models, conditional Ire1alpha (albumin-Cre//Ire1alphaflox/-) mice were employed in that study. This manuscript by Xhang et al. describes these mice in exquisite detail. Analysis of liver in development and after tunicamycin challenge shows that Ire1alpha deficiency produces very mild ER impairment and lipid accumulation in untreated mice, and confirms marked lipid accumulation/steatosis after tunicamycin challenge. Comprehensive transcriptomic analysis*

and biochemical analysis of the three arms of UPR show that after tunicamycin treatment, a persistant, maladaptive CHOP response is activated associated with hepatocyte apoptosis. Absence of Ire1alpha derepresses transcriptional activators of lipid biosynthesis and limits lipid secretion during ER stress and some of these perturbations were confirmed in cultured cells to rule out adaptive changes due to chronic deficiency of Ire1alpha. Finally, bortezomib-induced ER stress (a proteasome inhibitor that impairs ERAD) produced similar defects in the context of Ire1alpha deficiency.

The manuscript is clearly written, rich in data, beautifully illustrated, and appropriately interpreted, discussed and referenced.

Although the major findings of this paper initially confirm the prior study by the same group, or other single findings from other studies in other cultured cell types with ER stress, the comprehensive analysis of ER stress-induced hepatic steatosis in the absence of Ire1alpha allows special insights about how different arms of the UPR interact dynamically in both acute and chronic ER stress, and about what molecular events accompany the transition from adaptive to maladaptive ER stress response-all in tissue (not cultured fibroblasts). As a consequence, this paper will be of broad interest to the cell biology and misfolded protein disease communities.

Response: We thank this reviewer for the valuable comments. To further improve our manuscript, we performed additional experiments. The major additions to the revised manuscript include:

(1) Two pathophysiologic (non-TM) stress models, expression of a misfolding-prone human blood clotting factor VIII in the liver and partial hepatectomy, were included to further confirm the role of IRE1α in preventing stress-induced hepatic steatosis (Figure 8E-I and Supplemental figure 7).
(2) We provided more detailed information regarding the change of gene expression profile in the liver tissue of the *Ire1α*-null mice under the non-stressed condition (Supplemental figure 1). We included the result interpretation and discussion on mild steatosis observed in *Ire1α*-null liver under the non-stressed condition in the revised manuscript.

(3) We also examined expression of additional lipogenic regulators, including those involved in fatty acid oxidation and uptake/transport, in the *Ire1α*-null and control livers in the absence or presence of TM challenge (Supplemental figure 4). In particular, we clarified expression levels of SREBP-1c precursor and its mature form as well as differential expression of PPARγ1 and PPARγ2 in the *Ire1α*- null and control mouse liver in response to ER stress (Figure 5D and Supplemental figure 3). We discussed possible feedback regulation of gene expression and the inconsistency between gene expression and lipogenic phenotypes in ER stress-induced hepatic steatosis.

(4) We repeated the pulse-chase assay for secretion of ApoB lipoproteins with primary hepatocytes from *Ire1α*-null and control mice. In support of our original conclusion that IRE1α is required for apolipoprotein secretion, new pulse-chase assay results demonstrated that the secretion rate of ApoB lipoproteins from *Ire1α*-null primary hepatocytes was decreased, compared to that of control hepatocytes, in response to TM treatment (Figure 7C and D).

Specific suggestions:

1) the Yamamoto et al citation on page 5 is missing the year.

Response: We thank the reviewer for pointing this out. We have fixed the reference accordingly**.**

2) Some of the names or annotations are cropped by column lines in Table 1.

Response: We thank the reviewer for pointing this out. We have fixed the table 1 accordingly.

3) The significant differences between CTL and KO gene expression without TM injection might be marked with an asterisk to attract the reader's attention in Table 1.

Response: As suggested, we marked the significant difference between CTL and KO gene expression (without TM) with an asterisk in table 1. Moreover, in the revised manuscript, we

provided additional information for the genes whose expression was decreased or increased in the *Ire1α*-null mice (compared to the control) without TM treatment (Supplemental figure 1A and B).

References

Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, Lennon CJ, Kluger Y, Dynlacht BD (2007) XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. *Mol Cell* **27:** 53-66

Dubuquoy L, Dharancy S, Nutten S, Pettersson S, Auwerx J, Desreumaux P (2002) Role of peroxisome proliferator-activated receptor gamma and retinoid X receptor heterodimer in hepatogastroenterological diseases. *Lancet* **360:** 1410-1418

Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J (1997) The organization, promoter analysis, and expression of the human PPARgamma gene. *J Biol Chem* **272:** 18779-18789

Fausto N (2001) Liver regeneration: from laboratory to clinic. *Liver Transpl* **7:** 835-844

Fausto N, Campbell JS, Riehle KJ (2006) Liver regeneration. *Hepatology* **43:** S45-53

Fels DR, Ye J, Segan AT, Kridel SJ, Spiotto M, Olson M, Koong AC, Koumenis C (2008) Preferential cytotoxicity of bortezomib toward hypoxic tumor cells via overactivation of endoplasmic reticulum stress pathways. *Cancer Res* **68:** 9323-9330

Fribley A, Zeng Q, Wang CY (2004) Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. *Mol Cell Biol* **24:** 9695-9704

Gavrilova O, Haluzik M, Matsusue K, Cutson JJ, Johnson L, Dietz KR, Nicol CJ, Vinson C, Gonzalez FJ, Reitman ML (2003) Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *J Biol Chem* **278:** 34268-34276

Hernandez-Espinosa D, Minano A, Martinez C, Ordonez A, Perez-Ceballos E, de Arriba F, Mota RA, Ferrer F, Gonzalez M, Vicente V, Corral J (2008) Inhibition of proteasome by bortezomib causes intracellular aggregation of hepatic serpins and increases the latent circulating form of antithrombin. *Lab Invest* **88:** 306-317

Kaufman RJ, Pipe SW, Tagliavacca L, Swaroop M, Moussalli M (1997) Biosynthesis, assembly and secretion of coagulation factor VIII. *Blood Coagul Fibrinolysis* **8 Suppl 2:** S3-14.

Lee AH, Iwakoshi NN, Anderson KC, Glimcher LH (2003) Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. *Proc Natl Acad Sci U S A* **100:** 9946-9951

Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K, Kaufman RJ (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev* **16:** 452-466

Malhotra JD, Miao H, Zhang K, Wolfson A, Pennathur S, Pipe SW, Kaufman RJ (2008) Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci USA* **105:** 18525-18530

Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, Brewer B, Jr., Reitman ML, Gonzalez FJ (2003) Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest* **111:** 737-747

Miao HZ, Sirachainan N, Palmer L, Kucab P, Cunningham MA, Kaufman RJ, Pipe SW (2004) Bioengineering of coagulation factor VIII for improved secretion. *Blood* **103:** 3412-3419

Musso G, Gambino R, Cassader M (2009) Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Prog Lipid Res* **48:** 1-26

Nawrocki ST, Carew JS, Dunner K, Jr., Boise LH, Chiao PJ, Huang P, Abbruzzese JL, McConkey DJ (2005) Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells. *Cancer Res* **65:** 11510-11519

Ota T, Gayet C, Ginsberg HN (2008) Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. *J Clin Invest* **118:** 316-332

Qiu W, Su Q, Rutledge AC, Zhang J, Adeli K (2009) Glucosamine-induced endoplasmic reticulum stress attenuates apolipoprotein B100 synthesis via PERK signaling. *J Lipid Res* **50:** 1814-1823

Rosinol L, Montoto S, Cibeira MT, Blade J (2005) Bortezomib-induced severe hepatitis in multiple myeloma: a case report. *Arch Intern Med* **165:** 464-465

Rutkowski DT, Wu J, Back SH, Callaghan MU, Ferris SP, Iqbal J, Clark R, Miao H, Hassler JR, Fornek J, Katze MG, Hussain MM, Song B, Swathirajan J, Wang J, Yau GD, Kaufman RJ (2008) UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Dev Cell* **15:** 829-840

Rutledge AC, Qiu W, Zhang R, Kohen-Avramoglu R, Nemat-Gorgani N, Adeli K (2009) Mechanisms targeting apolipoprotein B100 to proteasomal degradation: evidence that degradation is initiated by BiP binding at the N terminus and the formation of a p97 complex at the C terminus. *Arterioscler Thromb Vasc Biol* **29:** 579-585

Schadinger SE, Bucher NL, Schreiber BM, Farmer SR (2005) PPARgamma2 regulates lipogenesis and lipid accumulation in steatotic hepatocytes. *Am J Physiol Endocrinol Metab* **288:** E1195-1205

Tontonoz P, Graves RA, Budavari AI, Erdjument-Bromage H, Lui M, Hu E, Tempst P, Spiegelman BM (1994) Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR gamma and RXR alpha. *Nucleic Acids Res* **22:** 5628-5634

Vidal-Puig AJ, Considine RV, Jimenez-Linan M, Werman A, Pories WJ, Caro JF, Flier JS (1997) Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest* **99:** 2416-2422

Yamazaki T, Shiraishi S, Kishimoto K, Miura S, Ezaki O (2010) An increase in liver PPARgamma2 is an initial event to induce fatty liver in response to a diet high in butter: PPARgamma2 knockdown improves fatty liver induced by high-saturated fat. *J Nutr Biochem.* PMID: 20801631 (in press)

Zhang J, Fu M, Cui T, Xiong C, Xu K, Zhong W, Xiao Y, Floyd D, Liang J, Li E, Song Q, Chen YE (2004) Selective disruption of PPARgamma 2 impairs the development of adipose tissue and insulin sensitivity. *Proc Natl Acad Sci U S A* **101:** 10703-10708

Zhang K, Wong HN, Song B, Miller CN, Scheuner D, Kaufman RJ (2005) The unfolded protein response sensor IRE1alpha is required at 2 distinct steps in B cell lymphopoiesis. *J Clin Invest* **115:** 268-281

Zhang YL, Hernandez-Ono A, Siri P, Weisberg S, Conlon D, Graham MJ, Crooke RM, Huang LS, Ginsberg HN (2006) Aberrant hepatic expression of PPARgamma2 stimulates hepatic lipogenesis in a mouse model of obesity, insulin resistance, dyslipidemia, and hepatic steatosis. *J Biol Chem* **281:** 37603-37615

2nd Editorial Decision 28 January 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have done significant additional work, including examining two non-TM models of ER stress. There are still some incompletely answered questions but that is the nature of science.
This is an outstanding effort and a significant work that should be published.

Referee #2 (Remarks to the Author):

Zhang and colleagues have provided evidence that general ER homeostasis, and not the activity of specific targets of one or the other UPR stress sensors, is required to avoid hepatic steatosis. In this revision, they have strengthened their original observations based on Ire1alpha deficient liver in which UPR was activated with tunicamycin. They have confirmed the same observations in a more physiological, non-pharmacological model of ER stress in liver due to retention of the misfolding prone human Factor VIII.

The authors have responded extensively to all of the reviewers' criticisms with several new experiments and reasonable responses. The manuscript is significantly improved.

