Manuscript NO.: 74751

Title: Family with Sequence Similarity 134 Member B-Mediated Reticulophagy Ameliorates Hepatocyte Apoptosis Induced by Dithiothreitol

Dear Editor:

We would like to take this opportunity to thank the editors and reviewers for their constructive suggestions. Accordingly, we have made the necessary changes to the manuscript to acknowledge the concerns of the editors and reviewers, as well as to fully meet the requirements of the publishing policies of *World Journal of Gastroenterology*. As a result, we believe that the quality of the manuscript is greatly strengthened. All amendments are highlighted in red in the revised manuscript. In addition, point-by-point responses to the comments are listed after this letter. The comments are copied in black and italics, while our responses are in red font.

We hope that it is now acceptable for publication in your journal, and we look forward to hearing from you soon.

Yours sincerely,

Dr. Rujia Xie
Corresponding author

First of all, we would like to express our sincere gratitude to the reviewers for their helpful and valuable comments.

Replies to Reviewer 1

Specific Comments

Comments

1. In the abstract, the ER-resident protein called 'family with sequence similarity 134 member B' (FAM134B), that can form a complex with calnexin (CNX) and microtubule-associated protein LC3II should be shortly introduced to the reader as protein which can mediate the selective isolation of ER fragments.

Response: Thank you for your valuable suggestion. We have added relevant introduction in the abstract according to the comment in the revised manuscript.

2. Results: in the end of the second paragraph the conclusion should be changed: Thus, our results revealed that expression of FAM134B is induced in response to ER stress.

Response: Thank you for your valuable suggestion. We have changed this expression according to the comment in the revised manuscript.

3. Results: in the begin of the third paragraph a reference should be given: It has been shown that FAM134B may interact with CNX in the cytosol or ER membrane (REFERENCE).

Response: Thank you for your careful review. We have cited one reference in the revised manuscript.

Forrester A, De Leonibus C, Grumati P, Fasana E, Piemontese M, Staiano L, Fregno I, Raimondi A, Marazza A, Bruno G, Iavazzo M, Intartaglia D, Seczynska M, van Anken E, Conte I, De Matteis MA, Dikic I, Molinari M, Settembre C. A selective ER-phagy exerts procollagen quality control via a Calnexin-FAM134B complex. *EMBO J* 2019; **38**(2): e99847 [PMID: 30559329 DOI: 10.15252/embj.201899847]

4. In order to form the CANX-FAM134B-LC3B how is the role of the chaperone calnexin? Do you have data argue for calnexin as a core protein or is this role more given by FAM134B?

Response: Thank you for your valuable suggestion. CANX is a molecular chaperone that assists the folding of monoglucosylated glycoprotein in the ER. CANX forms transient but relatively stable complexes with unfolded ER proteins until they either become folded or are degraded. FAM134B is not predicted to have an ER luminal domain, so FAM134AB indirectly interact with luminal proteins via a chaperone CNX. In a previous report, the authors found that the CANX-FAM134B interplay is devoted to the degradation of procollagen.

5. The data could be further validated by the use of another cell line or a functional model. Do you think the molecular findings are characteristic for hepatocytes? Both points should be addressed in the Discussion.

Response: This question is interesting. Firstly, the main reason for the use of rat normal hepatocyte BRL-3A to establish a cell model is that it has been found that human hepatocyte LO2 and WRL-68 have been contaminated with Hela cells, and if the appropriate cell line can be purchased, it can be further verified on the human cell model. Secondly, we are planning a study that aims to demonstrate whether FAM134B-mediated ER-phagy has the same effect in animal model. However, since the in vivo study takes a long period of time, we would like to publish the in vitro study with the molecular mechanisms as an independent paper.

ER-phagy is one of the most critical quality control mechanisms for the ER in multiple cell types. Defects in ER-phagy pathways are associated with multiple human pathologies, including infectious and neurodegenerative diseases, aging and cancer. However, the precise regulatory mechanisms in hepatocyte apoptosis remain unclear.

Replies to Reviewer 2

Specific Comments

Major issues:

1. In experimental design, a rational to use DTT as an inducer of ER stress-caused apoptosis of hepatocytes need to be explained. Major drawback is to use only rat cell line BRL-3A to draw a conclusion. Human hepatocyte cell line might serve as a model physiologically relevant to human more

than rat hepatic cell.

Response: Thank you for your valuable suggestion. For the first comment, the role of DTT is to inhibit the formation of disulfide bonds and lead to the accumulation of unfolded proteins in the ER, which leads to ER stress. If the ER stress cannot be alleviated, aberrant ER stress can trigger cell apoptosis. For the second comment, the main reason for the use of rat normal hepatocyte BRL-3A to establish a cell model is that it has been found that human hepatocyte LO2 and WRL-68 have been contaminated with Hela cells, and if the appropriate cell line can be purchased, it can be further verified on the human cell model.

2. A. Introduction: some explanations in the result part can move to introduction.

Response: Thank you for your careful review. We re-wrote the sentence in the revised manuscript.

1) GRP78 is a prominent ER molecular chaperone, and CNX is a membrane-bound lectin protein in the ER that can increase the protein folding capacity (Ref).

Response: The relevant literature has been added to the revised manuscript.

Kopp MC, Larburu N, Durairaj V, Adams CJ, Ali MMU. UPR proteins IRE1 and PERK switch BiP from chaperone to ER stress sensor. *Nat Struct Mol Biol* 2019; **26**(11): 1053-1062. [PMID: 31695187 DOI: 10.1038/s41594-019-0324-9]

Kozlov G, Gehring K. Calnexin cycle - structural features of the ER chaperone system. *FEBS J* 2020; **287**(20): 4322-4340. [PMID: 32285592 DOI: 10.1111/febs.15330]

2) Even though the excessive build-up of misfolded or unfolded proteins can be alleviated via ER stress, previous studies reported that a selective autophagic pathway defined as ER-phagy can also be activated by ER stress to restore ER homeostasis (Ref).

Response: The relevant literature has been added to the revised manuscript.

Bernales S, McDonald KL, Walter P. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol* 2006; **4**(12): e423. [PMID: 17132049 DOI: 10.1371/journal.pbio.0040423]

Bernales S, Schuck S, Walter P. ER-phagy: selective autophagy of the endoplasmic reticulum. *Autophagy* 2007; **3**(3): 285-7. [PMID: 17351330 DOI: 10.4161/auto.3930]

2. B. Methods:

1) To allow others reproduce the data, provide dilutions of all antibodies used in the study.

Response: Thank you for your valuable suggestion. As suggested by the reviewer, we have added dilutions of all antibodies used in the study.

2) Provide method for measurement of protein concentration. What used as standard?

Response: The protein concentrations were determined by the bicinchoninic acid kit (Solarbio Science, Beijing, China), and known concentrations of BSA were used as standard.

3) Taking confocal image at each time points is not regarded as liver imaging. Confocal images need nuclear stain to indicate cells. Moreover, higher magnification or zoom-in mode will improve quality of data. Add scale bars. Which program was used to visualize images? Any image modification that will significantly alter quality of images, i.e., contrast adjustment.

Response: Thank you for your valuable suggestion. Our point-by-point responses are presented as followed.

For the first comment, in our attempt to stain the nucleus with hochest, we found that the red fluorescence signal of the ER around the nucleus would overlap with the blue fluorescence signal of the nucleus, thus interfering with our observation of the co-localization of ER with lysosomes, so we abandoned the nucleus staining.

For the second comment, all pictures had a maximum magnification（×200）. The figures inserted into Word will reduce the pixel, resulting in the figures are not clear, but we can provide original figures with pixels as 300dpi to improve the quality of the figures. The scale bars have been added in the revised manuscript.

For the third comment, images were visualized with Zeiss LSM Image Browser.

For the fourth comment, contrast adjustment to change image quality was done with photoshop.

4) Provide sequences of siRNA

Response: We have provided the sequences of siRNA in the revised manuscript.

5) Make sure the appropriate use of statistic. The t-test is a method that determines whether two populations are statistically different from each other, whereas ANOVA determines whether three or more populations are statistically different from each other.

Response: We apologize for the statistical analysis problems in the original manuscript. To be more clearly and in accordance with the reviewer concerns, more detailed statistical analysis was improved on page. All statistical analysis was performed with GraphPad Prism 7 and graphs generated through Microsoft Excel. Data are expressed as the mean ± standard deviation.Since all of our experimental

groups were larger than 3 groups, One-way ANOVA followed by Tukey's post hoc test was performed to analyze comparisons among multiple groups, a significant difference was considered as $P<0.05$.

3. C. Results

1) DTT-mediated ER stress triggers ER-phagy mediated by FAM134B in BRL-3A cells

1.1. Add references for "GRP78 is a prominent ER molecular chaperone, and CNX is a membrane-bound lectin protein in the ER that can increase the protein folding capacity (Ref)."

Response: Thank you for your careful review. We have cited two references in the revised manuscript.

Kopp MC, Larburu N, Durairaj V, Adams CJ, Ali MMU. UPR proteins IRE1 and PERK switch BiP from chaperone to ER stress sensor. *Nat Struct Mol Biol* 2019; **26**(11): 1053-1062. [PMID: 31695187 DOI: 10.1038/s41594-019-0324-9]

Kozlov G, Gehring K. Calnexin cycle - structural features of the ER chaperone system. *FEBS J* 2020; **287**(20): 4322-4340. [PMID: 32285592 DOI: 10.1111/febs.15330]

1.2. Provide un-cropped images as supplemental data

Response: Thank you for your valuable suggestion. We have provided the uncropped membranes in the supplement.

1.3. Add references for "Even though the excessive build-up of misfolded or unfolded proteins can be alleviated via ER stress, previous studies reported that a selective autophagic pathway defined as ER-phagy can also be activated by ER stress to restore ER homeostasis (Ref)."

Response: Thank you for your careful review. We have cited two references in the revised manuscript.

Bernales S, McDonald KL, Walter P. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol* 2006; **4**(12): e423. [PMID: 17132049 DOI: 10.1371/journal.pbio.0040423]

Bernales S, Schuck S, Walter P. ER-phagy: selective autophagy of the endoplasmic reticulum. *Autophagy* 2007; **3**(3): 285-7. [PMID: 17351330 DOI: 10.4161/auto.3930]

1.4. No data show ER-phagy mediated by FAM134B in this section. All data show upregulation of ER stress concomitantly to increased level of ER-phagy-related proteins (FAM134B, ATG12, and LC3).

Response: Thank you for your comment. FAM134B, an ER-anchored protein, was recently proposed as a major mammalian receptor for ER-phagy. Previous studies have reported that FAM134B contains an LC3-interacting region that can interact with LC3 protein to form autophagosomal membranes, leading

to efficient ER sequestration into an autophagosomal lumen. In addition, we further performed the interaction between FAM134B and LC3 via immunoprecipitation.

1.5. Heading of "DTT-mediated ER stress triggers ER-phagy mediated by FAM134B in BRL-3A cells" overwhelms the data. Given a lack of ER-phagy, this heading shall be changed to "DTT-mediated ER stress increase/upregulate ER-phagy-related FAM134B in BRL-3A cells".

Response: Thank you for the title suggested. The title in the revised manuscript has been replaced, becoming"DTT-mediated ER stress upregulate ER-phagy-related FAM134B in BRL-3A cells".

1.6. Add reference for "It has been shown that FAM134B may interact with CNX in the cytosol or ER membrane (Ref)."

Response: Thank you for your careful review. We have cited one reference in the revised manuscript.

Forrester A, De Leonibus C, Grumati P, Fasana E, Piemontese M, Staiano L, Fregno I, Raimondi A, Marazza A, Bruno G, Iavazzo M, Intartaglia D, Seczynska M, van Anken E, Conte I, De Matteis MA, Dikic I, Molinari M, Settembre C. A selective ER-phagy exerts procollagen quality control via a Calnexin-FAM134B complex. *EMBO J* 2019; **38**(2): e99847 [PMID: 30559329 DOI: 10.15252/embj.201899847]

1.7. It is unclear to me for the sentence of "There is an indirect interaction between FAM134B and lumenal proteins through the lumen-resident segment, which has chaperone activity in CNX." To my understand, CNX is membrane-bound lectin protein. Or do you mean "FAM134AB indirectly interact with luminal proteins via a chaperone CNX". Please clarify.

Response: Thank you for your suggestion. FAM134B is not predicted to have an ER luminal domain, so we mean "FAM134AB indirectly interact with luminal proteins via a chaperone CNX".

1.8. As a summary of section, "Collectively, these results suggest that ER-phagy mediated by FAM134B sequesters stressed ER membranes via a CANX-FAM134B-LC3B complex". There was no evidence of ER-phagy here. The ER stress-induced increase of FAM134B and interaction of CANX-FAM134B-LC3B only indicate possibility of ER-phagy. Without the ER-phagy data, please avoid overwhelmed conclusion.

Response: Thank you for underlining this deficiency. We have removed this sentence in the revised manuscript.

2) Endolysosomal delivery of ER is gradually blocked, but relieved in BRL-3A cells treated with DTT

long-term

2.1. To avoid confusion, please consistently use ER autolysosome.

Response: Thank you for underlining this deficiency. We have modified this expression according to the comment. “Endolysosomal” was changed to “ER autolysosome”.

2.2. As mentioned above, higher magnification or zoom-in mode of confocal images would allow co-local ization. Use arrows or arrow heads to indicate ER-localized lysosomes. Also, nuclear stain is important to define a cell, but not artifacts.

Response: For the first comment, all pictures had a maximum magnification（×200）. The figures inserted into Word will reduce the pixel, resulting in the figures are not clear, but we can provide original figures with pixels as 300dpi to improve the quality of the figures.

For the second comment, we have used arrows to indicate ER-localized lysosomes in the revised figures. In our attempt to stain the nucleus with hochest, we found that the red fluorescence signal of the ER around the nucleus would overlap with the blue fluorescence signal of the nucleus, thus interfering with our observation of the co-localization of ER with lysosomes, so we abandoned the nucleus staining.

2.3. According to “Notably, the cells treated for 48 h were targeted to the lysosomes at higher rates compared to those treated for 24 h (Fig. 2)”, which panels of Fig. 2 indicate higher rate of lysosome targeting?

Response: We are extremely grateful to reviewer for pointing out this problem. We have modified this expression. The modified expression was as followed. “Notably, the colocalization of ER and lysosomes in BRL-3A cells treated with DTT for 48 h was increased compared to those treated for 24 h. (Fig. 2)”

3) ER-phagy mediated by FAM134B induces mitochondrial calcium uptake at early time points, but mitochondrial calcium uptake is reduced after prolonged DTT treatment

3.1. To emphasize on FAM134B-mediated ER-phagy causing an increase of calcium uptake in mitochondria, knockdown of FAM134B is required. Moreover, level of calcium in Mt should be presented in quantitative manner, i.e., use of ImageJ to analyses fluorescence intensity.

Response: Thank you for the title suggested. The title in the revised manuscript has been replaced, becoming “Short-term DTT treatment induces mitochondrial calcium uptake while prolonged DTT treatment reduces it”. We have provided the Pearson’s coefficient of the colocalization of mitochondria and calcium using bar chart in the supplement.

3.2. Use italic for gene names. Also follow gene symbol for species.

Response: Thank you for your valuable suggestion. We have modified this expression throughout the text according to the comment.

4) DTT treatment induces cell cycle arrest and apoptosis of BRL-3A cells, which is relieved at 48 h

4.1. Fig. 4A, provide FSC, SSC and way to gate cell for analysis. Which quadrant represent apoptotic cells?

Response: Thank you for your valuable suggestion. We have provided the graph for FSC-SSC, the blank control group, PI group and FITC-Annexin V group in the supplement. We draw the gate cell for analysis according to blank control group, PI group and FITC-Annexin V group.

In Fig.4A, the lower right quadrant represents the early apoptotic cells and the upper right quadrant represents the late apoptotic cells.

4.2. Fig. 4C, show gate of S-phase. Gating for G1 and G2 phase is too small. Given three experiment performed, a statistic analysis of cell cycle should be calculated.

Response: Thank you for your valuable suggestion. We have provided the table for a statistic analysis of cell cycle and the modified graph for cell cycle in the revised manuscript.

5) BRL-3A cells undergo apoptosis upon FAM134B knockdown

5.1. *To indicate ER-phagy, ER autolysosome change must be examined*

Response: Thank you for your valuable suggestion. Since there are six ER-phagy receptors, FAM134B knockdown does not completely block ER autophagy process. Thus, we did not detect the ER autolysosome change.

5.2. Fig. 4B show that 24-h DTT induce apoptosis. In Fig. 5E-H, Need untreated control to verify that DTT still able to induce apoptosis.

Response: Thank you for your valuable suggestion. The first group in Fig. 5E-H showed that DTT can induce apoptosis in untreated cells.

5.3. There is controversy in Fig. 5Gand Fig. 4A. Fig. 4A, the 24-h DTT-treatment show apoptotic cells. But, here lower percentage of apoptosis was observed in Fig. 5G. Discussion on this controversy might help.

Response: In Fig. 5G and Fig. 4A, the apoptotic rate of DTT treatment for 24 h was 20 %, the result is not controversy.

5.4. To draw a conclusion in which FAM134B-mediated ER-phagy in the DTT-treated cells, marker of ER-phagy (ER autolysosome) need to be demonstrated. Bar graph of Fig. 5G-H indicates a synergistic effect of DTT and FAM134B knockdown on apoptosis, implying cytoprotective effect of FAM134B likely via ER-phagy if data support.).

Response: For the first comment, we used the colocalization of endoplasmic reticulum with lysosomal to reflect the ER autolysosome change.

For the second comment, lacking FAM134B will lead to the dysfunction of ER, thereby aggravating DTT-triggered hepatocyte apoptosis.

5.5. *Flow cytometry profile suggest inadequate compensation of the emitted fluorescence of PI and FITC, as suggested by pseudo color dots arranging as a line.*

Response: Thank you for your valuable suggestion. We have provided the new figures in the revised manuscript.

Replies to Reviewer 3

Specific Comments

1. How many cultures (biological and technical replicates) were studied for each exposure period?

Response: Images shown are representative of experiments carried out at least three times. Each sample was repeated 3 times or at least three dishes of cells in each group.

2. The authors indicate that they used one-way analysis of variance to identify statistically significant differences. However, this criterion assumes the distribution of features corresponding to the normal distribution law. Did the authors check the distributions in the sample and by what criterion?

Response: Thank you for your valuable suggestion. We have used the Shapiro-Wilk normality test to test the normal distribution of the data and all the data were fit to a normal distribution law.

3. In the case of a Western blot, uncropped membranes with mass markers must be included in the Supplement.

Response: We have provided the uncropped membranes in the supplement.

4. For confocal microscopy (Figure 2, 3), it is necessary to provide quantitative data, such as the collocalization coefficient, as well as a statistical analysis of the data obtained?

Response: Thank you for your valuable suggestion. We have provided the Pearson's coefficient of the colocalization of the data in Fig 2,3.

Replies to Science editor

This manuscript investigated the role of FAM134B in ER stress-induced endoplasmic reticulophagy (ER-phagy) and apoptosis in hepatocytes. The utility and data of the in vitro models employed in this manuscript do not adequately describe the title of this manuscript. The rationale for using DTT as an inducer of ER stress-induced hepatocyte apoptosis needs to be justified in the experimental design, the use of the rat cell line BRL-3A alone is not sufficient to draw conclusions, and human hepatocytes may serve as a more physiologically relevant model than rat hepatocytes.

Response: Thank you for your valuable suggestion. For the first comment, the role of DTT is to inhibit the formation of disulfide bonds and lead to the accumulation of unfolded proteins in the ER, which leads to ER stress. If the ER stress cannot be alleviated, aberrant ER stress can trigger cell apoptosis. For the second comment, the main reason for the use of rat normal hepatocyte BRL-3A to establish a cell model is that it has been found that human hepatocyte LO2 and WRL-68 have been contaminated with Hela cells, and if the appropriate cell line can be purchased, it can be further verified on the human cell model.

Replies to **Company editor-in-chief**

I have reviewed the Peer-Review Report, the full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office's comments and the

Criteria for Manuscript Revision by Authors. Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, "Figure 1Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...". Please provide decomposable Figures (in which all components are movable and editable), organize them into a single PowerPoint file. In order to respect and protect the author's intellectual property rights and prevent others from misappropriating figures without the author's authorization or abusing figures without indicating the source, we will indicate the author's copyright for figures originally generated by the author, and if the author has used a figure published elsewhere or that is copyrighted, the author needs to be authorized by the previous publisher or the copyright holder and/or indicate the reference source and copyrights. Please check and confirm whether the figures are original (i.e. generated de novo by the author(s) for this paper). If the picture is 'original', the author needs to add the following copyright information to the bottom right-hand side of the picture in PowerPoint (PPT): Copyright ©The Author(s) 2022.

Response: Thank you for your valuable suggestion. We have provided the decomposable Figures and organized them into a single PowerPoint file.