EMBO reports

PPARα Alleviates Iron Overload-induced Ferroptosis in Mouse Liver

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DOI: 10.15252/embr.202052280

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Review Timeline:

Submission Date:	15th Dec 20
Editorial Decision:	18th Dec 20
Revision Received:	22nd Dec 20
Editorial Decision:	5th Mar 21
Revision Received:	5th Dec 21
Editorial Decision:	15th Feb 22
Revision Received:	29th Apr 22
Editorial Decision:	20th May 22
Revision Received:	23rd May 22
Accepted:	23rd May 22

Editor: Martina Rembold

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **18th Dec 2020**

Dear Prof. Zhang

Thank you for the submission of your research manuscript to our editorial offices. I have now read and discussed it with the editorial team and we all agree that it might make a nice contribution to EMBO reports. We have therefore decided to send it out for peer review.

Before doing so, I however kindly ask you to make a minor revision to your manuscript. We noticed a related study, published in April 2020 (PMID 32079652), that reported on a role for PPARalpha in promoting ferroptosis downstream of MDM2 and MDMX. We feel that it is good scientific practice to discuss related and also opposing findings, and I therefore kindly ask you to include a short discussion of these results in your manuscript.

Once you have revised your study, please upload it using the following link and we will proceed with peer review.

Link Not Available

I look forward to seeing a revised form of your manuscript when it is ready.

Your sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

Dear Dr. Rembold,

We add more dissussion on the role of "PPARalpha in promoting ferroptosis downstream of MDM2 and MDMX", and resubmit the manuscript.
We all verified that the PPARα are related the ferroptosis, but there're some differences on the outcome of PPARα activity. The following points should be addressed. Firstly, most our work used in vivo mouse model, while Venkatesh et al used in vitro cell lines（Venkatesh, 2020）. Secondly, agonists of the PPARα were different. Moreover, PPARα were regulated by MDM2 only under certain conditions, i.e. clofibrate treatment(Gopinathan, 2009). Injury induced by this chemical is quite different with the overdose Fe treatment. All the three items might be the different ferroptosis outcome of PPARα activation.
There is the other possibility, that is, MDM2 promotes ferroptosis when PPARα activation, meanwhile, PPARα induced the TRF and GPX4 expression to keep the balance and make the body heathy. As we previously reported that PPARα makes contribution to homeostasis and protect the cells from over-toxin caused injury(Cheng, 2017).

Thank you for your time!

Merry christmas and happy holidays!

Sincerely yours,

Lisheng

1st Revision - Editorial Decision **5th Mar 2021**

Dear Prof. Zhang

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in handling your manuscript but we have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that you analyse ferroptosis and the role of PPARalpha in vivo, but they also raise important concerns regarding the conclusiveness of the data. Both referees point out that the causal link to GPX4 is not strong and that the role of other PPARalpha targets has not been tested. Referee 1 provided further feedback and emphasized once more that the potential contribution of AIFM2/FSP1 needs to be tested. Also a potential role of MDM2, MDMX and a contribution from NRF2 will need to be investigated.

From the referee comments it is clear that, as it stands, the data are too preliminary and publication of the manuscript in our journal can therefore not be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

We invite you to submit your manuscript within three months of a request for revision. This would be June 5th in your case. I should however emphasize two points here: (1) We are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. (2) I note that a significant amount of additional experimental work will be required to address all referee concerns. Therefore, if you need more time to complete these experiments and the revisions, please do not hesitate to contact me to discuss the time needed and the revisions further.

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IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.
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When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) Regarding data quantification
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
- Please also include scale bars in all microscopy images.

11) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

Review of manuscript EMBOR-2020-52280V2 -PPARα Alleviates Iron Overload-induced Ferroptosis in Mouse Livers. In the present study, the authos utilize a model of iron overload to induce ferroptosis like cell death and mice death by a mechanism potentially involving acute liver failure. The authors demonstrate that mice lackin PPARa are more sensitive to iron overload induced liver failure by a mechanism iron mishandling and lack of upregulation of the ferroptosis regulator GPX4.
The main strength of the paper is that it was mostly performed in vivo and this is definitely something the field of "ferroptosis" is lacking, strong experimental evidence for its pathological role. Having said this, the overall data is not impressive, and the connection between PPARa and GPX4 expression is weak and can hardly justify the protective effect observed, at leas is unlikely.
Rather the authors should have address another axis protecting from ferroptosis, namely the AIFM2/FSP1 system. Recent reports have already suggested that AIFM2/FSP1 can be induced in a PPARa dependent manner, and this should be highly relevant here.
Please see: MDM2 and MDMX promote ferroptosis by PPARα-mediated lipid remodelling. Venkatesh D, O'Brien NA, Zandkarimi F, Tong DR, Stokes ME, Dunn DE, Kengmana ES, Aron AT, Klein AM, Csuka JM, Moon SH, Conrad M, Chang CJ, Lo DC, D'Alessandro A, Prives C, Stockwell BR. Genes Dev. 2020 Apr 1;34(7-8):526-543.
Moreover, was not carefully assembled. References are misplaced and wrongly marked. The text sometimes lacks a logical flow, and some sentenced ended abruptly making the assessment and reading difficult. In the acknowledgement, I would like to point out that it's excellent to appreciate your colleague's contribution, but this is not a thesis.
As such, I find the paper overall premature and far away from being ready to be submitted.

Referee #2:

In this study, Xing and colleagues investigated the role of PPARα in regulating liver damage caused by a high-iron diet or dextriferron. First, they found that PPARα ligands (GW7647 or WY14643) can reduce liver damage caused by iron overload, which is related to the reduction of lipid peroxides, GSH and ROS. Second, they used molecular biology to show that GPX4 and TRF are potential target genes of PPARα, which may affect the sensitivity of ferroptosis. Finally, they observed that mice lacking PPARα were more sensitive to liver damage caused by a high-iron diet. In general, unlike previous findings that PPARα is a promoter of ferroptosis in vitro, these findings indicate that PPARα has a different effect on preventing ferroptosis in vivo. Although this topic is interesting, other data and control experiments are needed to support the author's conclusions.
1) Since previous studies have shown that MDM2 and MDMX-related ferroptotic death require PPARα, the authors need to test these pathways in parallel in their models.
2) There are many target genes of PPARα. How to confirm the up-regulation of GPX4 or the down-regulation of TFR is crucial for PPARα-mediated ferroptosis resistance? In other words, can overexpression of GPX4 or knockdown or TFR alleviate ferroptosis caused by PPARα deficiency?
3) PPARα plays an important role in liver biology. Another important model of ferroptosis in the liver is the use of sorafenib. In particular, the activation of NRF2 pathway diminishes sorafenib-induced ferroptosis in vitro and in vivo (PMID: 26403645). More importantly, GPX4 and TFR are also target genes of NFR2 (PMID: 32123318). Is there an interaction between the PPARα and NRF2 pathways in liver?
4) Although the authors tested PTGS2 as a so-called marker of ferroptosis, the up-regulation of PTGS2 is a common event of inflammation. In addition to PTGS2, the author also needs to monitor apoptosis markers (such as TUNEL) or cell death-related DAMPs, especially HMGB1 (PMID: 30686534). Otherwise, it is difficult to distinguish between ferroptotic and non-ferroptotic death in mice on a high-iron diet.

We deeply appreciate the time and effort you've spent in reviewing our revision manuscript entitled " EMBOR-2020-52280V2- PPARα Alleviates Iron Overload–induced Ferroptosis in Mouse Livers through GPX4". Your comments are constructive and helpful. We have carefully revised the manuscript according to reviewer's suggestion and made point to point response.

Reviewer #1 (Comments for the Author:):

1. The main strength of the paper is that it was mostly performed in vivo and this is definitely something the field of "ferroptosis" is lacking, strong experimental evidence for its pathological role. Having said this, the overall data is not impressive, and the connection between PPARα and GPX4 expression is weak and can hardly justify the protective effect observed, at least is unlikely. Rather the authors should have addressed another axis protecting from ferroptosis, namely the AIFM2/FSP1 system. Recent reports have already suggested that AIFM2/FSP1 can be induced in a PPARα dependent manner, and this should be highly relevant here.

Reply: Following your suggestion, we examined expression of AIFM2/FSP1 in mouse liver during the development of HID-induced ferroptosis. But the expression of AIFM2/FSP1 was comparable between WT and PPAR knockout mouse liver. Gpx4 and FSP1 were both identified as ferroptosis suppression factors, and AIFM2 has been hereafter renamed ferroptosis suppressor protein-1 (FSP1) due to its critical role in a second FSP1–Q10–NADPH system, independent of the canonical GSH-based GPx4 pathway, which may regulate ferroptosis execution (Liu, 2020). It was reported in Venkatesh's paper that increased PPARα activity suppressed ferroptosis while decreased PPARα activity increased their sensitivity to ferroptosis in vitro (Venkatesh, 2020）. This is consistent with the results of our in vivo experiments. We also demonstrated that PPARα inhibited ferroptosis through direct regulation of GPX4 and TRF expression. For this reason, we speculate that PPARα-GPX4/TRF is the main signaling pathway in HID-induced ferroptosis.

2. Moreover, was not carefully assembled. References are misplaced and wrongly marked. The text sometimes lacks a logical flow, and some sentenced ended abruptly making the assessment and reading difficult. In the acknowledgement, I would like to point out that it's excellent to appreciate your colleague's contribution, but this is not a thesis.

Reply: Thank you for the advice. Following your suggestion, we've re-organized the results, illustrative logic and conclusion in the revision manuscript. The revision manuscript has been reviewed and edited by a native speaker of English.

Reviewer #2 (Comments for the Author:):

1. Since previous studies have shown that MDM2 and MDMX-related ferroptotic death require PPARα, the authors need to test these pathways in parallel in their models.

Reply: We examined the expression of MDM2 and MDMX throughout HID-induced ferroptosis. But expression of MDM2 and MDMX showed no significant changes. Venkatesh and we all verified that the increased PPARα activity suppressed ferroptosis while decreased PPARα activity increased their sensitivity to ferroptosis. The following points should be addressed. Firstly, most our work was performed in vivo mouse models, while Venkatesh et al used in vitro cell lines (Venkatesh, 2020). Secondly, different agonists of the PPARα were used, and the specificity and sensitivity may vary. Moreover, in our experiment, ferroptosis was inhibited by direct activation of PPARα, whereas in Venkatesh's paper, that lowered PPARα activity is a key conduit for MDM2/X to suppress the antioxidant defenses of cells and thereby promote ferroptosis. These results indicate that the activity of PPARα is one of the keys signaling to regulate ferroptosis.
There is the other possibility, that is, MDM2 promotes ferroptosis, meanwhile, PPARα promotes GPX4 expression and inhibits TRF expression to keep the balance and make the body heathy. As we previously reported that PPARα makes contribution to homeostasis and protects the cells from over-toxin caused injury (Cheng, 2017).

2. There are many target genes of PPARα. How to confirm the up-regulation of GPX4 or the down-regulation of TFR is crucial for PPARα-mediated ferroptosis resistance? In other words, can overexpression of GPX4 or knockdown or TFR alleviate ferroptosis caused by PPARα deficiency?

Reply: Thank you for the advice. We assessed the in vivo potential of GPX4 to prevent the ferroptosis in PPARα disruption animals, and found the expression of GPX4 was significantly decreased when the mice were fed a high-iron diet. PPARα-/- mice were injected with GPX4-AAV before the mice fed HID diet, and the ferroptosis was improved generally, indicating the effect of the overexpression GPX4 on protecting against iron-overload induced PPARα-/- mice liver injury. Mice with GPX4-AAV treatment significantly reduced risk of animal death compared to the control AAV group. These results suggest that the protective role of GPX4 against ferroptosis in PPARα-/- mouse liver injury model.

3. PPARα plays an important role in liver biology. Another important model of ferroptosis in the liver is the use of sorafenib. In particular, the activation of NRF2 pathway diminishes sorafenib-induced ferroptosis in vitro and in vivo (PMID: 26403645). More importantly, GPX4 and TFR are also target genes of NFR2 (PMID: 32123318). Is there an interaction between the PPARα and NRF2 pathways in liver?

Reply: Considering the Reviewer’s suggestion, we have detected expression of NRF2

and NRF2-related genes. But there was no significant association between PPARα and NRF2 in our model. Therefore, we think that PPARα and NRF2 pathways may be independent of each other in our model.

4. Although the authors tested PTGS2 as a so-called marker of ferroptosis, the up-regulation of PTGS2 is a common event of inflammation. In addition to PTGS2, the author also needs to monitor apoptosis markers (such as TUNEL) or cell death-related DAMPs, especially HMGB1 (PMID: 30686534). Otherwise, it is difficult to distinguish between ferroptotic and non-ferroptotic death in mice on a high-iron diet.

Reply: Apoptosis was detected using TUNEL assay. It didn't show significant differences in number of TUNEL positive cells between HID group and control mice. The data suggested that apoptosis might not be the main cause of liver damage in HID mice. We have detected expression of HMGB1 in the liver of HID mice. An increasing trend HMGB1 expression was found in mouse liver during HID. But GW7647 significantly repressed HMGB1 expression in HID mice suggesting the beneficial effects of PPARα in the hepatic cell death therapy. Experimental results consistent with previous reports.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.

Dear Prof. Zhang

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, both referees acknowledge that the revision has significantly improved the study but referee 2 also points out several concerns that have not been addressed. Please address points 1 and 2 by providing further experiments, please discuss the divergent role of PPARgamma in ferroptosis (point 3) and please ensure that the methods are detailed enough to allow other scientists to reproduce the experiments. This also applies e.g. to the identification and characterization of the fluorescent Fe2+ probe (see below).

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Your manuscript currently contains five figures and therefore qualifies to be published in our Reports section. In order to publish your study as Report we need you to combine the Results and Discussion section and please keep the limit of 25,000 (+/- 2,000) characters for the main text in mind (excluding references and materials and method).

- Figure 3: please remove the numbers above the scale bar from the figure panels. The numbers cannot be read and the size is defined in the legend anyway.

- Figure EV1G: please add a scale bar to the figure panel.

- Appendix: please combine the table of content, Appendix Figure S1 and Appendix table S1 into one single pdf document.

- Regarding the structure of the fluorescent Fe2+ specific probe: it was not clear to me whether you have used here a published probe or whether you have identified the probe yourself in the current study. Could you please clarify this? Neither the methods nor the results section clearly state this or give detailed information on the identity and identification of this sensor.

- Figure callouts: We noticed that Fig EV1 and EV3 panel callouts are not alphabetical nor numerically ordered, ie Fig EV1E comes after EV3C, and EV3A is after EV4D. We generally recommend arranging the figures so that the individual panels and the figures can be introduced in a numerical/alphabetical order.

- Please remove the abbreviations.

- Please order the individual manuscript sections as recommended in our guide to authors.

- Please remove the Graphical Abstract legend from the figure legends.

- I have also taken the liberty to make some changes to the Abstract (below my signature). Could you please review it?

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, and B) 2-3 bullet points highlighting key results. Please send us a draft for this text along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors have done a fair job at better contextualizing their findings and I don't have any additional comments at this stage besides that the citation on the role of FSP1 is incorrect, and instead of Liu, 2020 should be PMID 31634899 and 31634900

Referee #2:

Most of my comments have been addressed, but some remain unsatisfactorily answered.
1. The authors need to determine whether knockdown of MDM2 and MDMX affects PPARα activity and GPX4 expression. Otherwise, a simple expression analysis of MDM2/MDMX cannot exclude their contribution.
2. There is no published data showing that HMGB1 is up-regulated during ferroptosis. Conversely, HMGB1 release increases during ferroptosis. The authors need to examine the release of HMGB1.
3. Different PPAR family has different function. Unlike PPARα inhibiting ferroptosis in the present study, a recent study showed that PPARγ promotes ferroptosis (PMID: 34478917). The authors may need to discuss the potential implications of these findings.
4. The description of some methods is too simple.

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PPARα Alleviates Iron Overload-induced Ferroptosis in the Mouse Liver

ABSTRACT (please clarify whether you have developed the fluorescent probe or used and characterized a probe that was developed before)
Ferroptosis is an iron-dependent form of non-apoptotic cell death implicated in liver, brain, kidney, and heart pathology. How ferroptosis is regulated remains poorly understood. Here, we show that PPARα suppresses ferroptosis by promoting the expression of glutathione peroxidase 4 (Gpx4) and by inhibiting the expression of the plasma iron carrier TRF. PPARα directly induces Gpx4 expression by binding to a PPRE element within intron 3. PPARα knockout mice develop more severe iron accumulation and ferroptosis in the liver when fed a high-iron diet than wildtype mice. Ferrous iron (Fe^{2+}) triggers ferroptosis via Fenton reactions and ROS accumulation. To monitor Fe^{2+} we used a rhodamine-based "turn-on" fluorescent probe, which can be synthesized with high yield, displays high selectivity towards Fe^{2+}, and exhibits a stable response for Fe^{2+} with a concentration of 20 μM in tissue. Our data thus show that PPARα activation alleviates iron overload-induced ferroptosis in mouse livers through Gpx4 and TRF, suggesting that PPARα may be a promising therapeutic target for drug discovery in ferroptosis-related tissue injuries. Moreover, we identified a fluorescent probe that specifically labels ferrous ions and can be used in vivo studies to monitor the Fe^{2+}.

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "EMBOR-2020-52280V2- PPARα Alleviates Iron Overload-induced Ferroptosis in Mouse Livers". Those comments are all valuable and very helpful for revising and improving our paper. We have studied comments carefully and have made correction which we hope meet with approval. The main corrections in the paper and the responds to the reviewer's comments are as flowing:

Response to the reviewer's comments:

Reviewer #1:

1. The authors have done a fair job at better contextualizing their findings and I don't have any additional comments at this stage besides that the citation on the role of FSP1 is incorrect, and instead of Liu, 2020 should be PMID 31634899 and 31634900

Reply: Thank you for the advice. We are very sorry for our incorrect citation. Following your suggestion, we've re-organized the references in the revision manuscript.

Reviewer #2:

1. The authors need to determine whether knockdown of MDM2 and MDMX affects PPARα activity and GPX4 expression. Otherwise, a simple expression analysis of MDM2/MDMX cannot exclude their contribution.

Reply: It was reported that the MDM2–X complex might post-translationally modify PPARα and alter its activity(PMID: 19103650; PMID: 32079652). To further determine whether knockdown of MDM2 and MDMX affects PPARα activity and GPX4 expression, siRNAs were used to knock down MDM2 and MDMX in Hep1-6 cells, and then the expression of PPARα target genes CPT1 and Gpx4 was detected. Although not statistically significant, there was a trend of increasing of CPT1 expression after knockdown of MDM2 or MDMX. Knockdown of both MDM2 and MDMX resulted in a significant down-regulation of GPX4 expression. The result suggests that knockdown of MDM2 and MDMX enhanced the activity of PPARα, but suppressed the expression of Gpx4 in an unknown regulatory manner. It requires further research to explore the mechanism.

2. There is no published data showing that HMGB1 is up-regulated during ferroptosis. Conversely, HMGB1 release increases during ferroptosis. The authors need to examine the release of HMGB1.

Reply: Abundance of HMGB1 proteins in liver and serum of high-iron diet mice was determined, and we found that the content of HMGB1 in liver of the high iron diet group was significantly increased compared with control group, and the content of HMGB1 in serum showed a trend of increase. These results suggest that HMGB1 release increases in our ferroptosis model.

3. Different PPAR family has different function. Unlike PPARα inhibiting ferroptosis in the present study, a recent study showed that PPARγ promotes ferroptosis (PMID: 34478917). The authors may need to discuss the potential implications of these findings.

Reply: In our study, PPARα inhibits ferroptosis by promoting GPX4 expression and reducing TRF expression. And in the Hwang's paper， PAPRδ rescues xCT-deficient cells from ferroptosis by targeting peroxisomes (PMID: 34649350). Interestingly, Tao indicated CYP2J2-produced epoxyeicosatrienoic acids contribute to the ferroptosis resistance of pancreatic ductal adenocarcinoma in a PPARγ-dependent manner (PMID: 34707002), whereas Han showed that PPARγ drives ferroptosis in DCs (PMID: 34478917). These findings indicate that PPARs and PPAR agonists play an important role in the process of ferroptosis. Considering that PPAR isotypes are expressed in different tissues and have functional differences and similarities, targeting PPARs for future clinical therapy need more investigation on ferroptosis related diseases.

4. The description of some methods is too simple.

Reply: Thank you for the advice. Following your suggestion, we 've added more details on the methods' description including the identification and characterization of the fluorescent Fe^{2+} probe in the revision manuscript.

We tried our best to improve the manuscript and made some changes in the manuscript (bule or red font in the revision). These changes will not influence the content and framework of the paper.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

3rd Revision - Editorial Decision **20th May 2022**

Manuscript number: EMBOR-2020-52280V4
Title: PPARα Alleviates Iron Overload-induced Ferroptosis in Mouse Livers.
Author(s): Guowei Xing, Lihua Meng, Shiyao Cao, Shenghui Liu, Jiayan Wu, Qian Li, Wendong Huang, and Lisheng Zhang

Dear Prof. Zhang

Thank you for your patience while we have reviewed your revised manuscript. Referee #2 now also supports publication of your manuscript. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- Appendix: Please change 'QRT-PCR' to 'qRT-PCR' in Appendix Table S1 and please change "Figure S1" to "Appendix Figure S1" in the figure legend.

- I have taken the liberty to introduce some changes into the Abstract. Please review my suggestions in the attached document. I suggest to give the probe a name to enhance its recognition. If you decide to do so, please apply this name throughout the manuscript. I also suggest to have the manuscript proof-read again by a native speaking colleague.

- I have also introduced some changes to the summary text you sent. Please review these as well.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

Link Not Available

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

Referee #2:

They address experimentally.

The authors have addressed all minor editorial requests.

Prof. Lisheng Zhang
College of Veterinary Medicine, Huazhong Agricultural University
No.1 Shizishan Street
HuBei 430070
China

Dear Prof. Zhang,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Corresponding Author Name: Lisheng Zhang
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-52280V4

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Three trials were performed and at least 3 mice were used in each experiment group.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Randomization of the animal samples was used in the animal studies
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding were used in the animal studies.
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	YES . Statistical analyses were performed with GraphPad Prism 6.0 software using one-way ANOVA for multiple comparison or Mantel-Cox test for survival analyses.
Is there an estimate of variation within each group of data?	YES

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http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti-GPX4 (Abcam; no. 125066);PPARα antibodies (Santa Cruz; sc-398394);anti-TRF (Hangzhou HuaAn Biotechnology; R1212-1)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Hep1-6 cells were purchased from the National Infrastructure of Cell Line Resource (NICR, CHN).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57BL/6J SPF mice,PPARα−/− mice (strain name: B6;129S4-Pparαtm1Gonz/J, stock number 008154). Male mice between 6 and 8 weeks old were used in each group.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental protocols were approved by the animal ethical and welfare committee of Huazhong Agricultural University.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We consulted all of the guidelines and the compliance was confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	PI and IRE reviewed annualy and we carried out the experiments according to the DURC policies.