EMBO reports

Iron loss triggers mitophagy through induction of mitochondrial ferritin

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1st Editorial Decision 10th Mar 2020

Dear Prof. Hino,

Thank you for the submission of your research manuscript to our journal, which was now seen by three referees, whose reports are copied below.

As you can see, the referees express interest in the analysis. However, they also raise a number of concerns that need to be addressed to consider publication here. As for the remarks of referee #1, I agree that reorganizing the data as he/she suggested would increase the clarity and accessibility of the study to a broader audience - i.e. moving the in vivo data to the end. Please let me know if you have additional comments on this point.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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 providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter plots in these cases.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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.

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).

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. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
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."

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) 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 .

8) 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 .

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. 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 note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

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,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

In this manuscript, Hara and colleagues observed that iron depletion induced by Deferiprone (DFP) decreases hepatocellular carcinoma in two mouse models for non-alcoholic steatohepatitis (NASH). The authors show that this tumor decrease potentially correlates with mitophagy induction in vivo. Consistent with earlier work, the authors observe that DFP induces mitophagy in their in vitro cellular models. Interestingly, they found that upon iron loss, the expression of mitochondrial ferritin (FTMT) is increased by the action of two transcription factors: Hif1a and SP1, and that silencing of FTMT blocks mitophagy. The authors present data suggesting that FTMT is localized to the outer membrane of mitochondria where it interacts with the selective cargo receptor NCOA4, to drive DFP-induced mitophagy.
Overall, this is a very interesting story that could explain the molecular details of how iron availability regulates mitophagy - and this work is certainly publishable. There are vast amounts of data in this manuscript, in relation to both in vivo studies and in vitro work, and unfortunately this makes reading the manuscript a little hard to digest (it was easy to get lost in the numerous figure panels and supplementary figures). I feel the real strength of the current manuscript lies in the molecular work, while the in vivo work - though very encouraging, is only correlative with respect to mitophagy actually suppressing HCC and not some other consequence of iron loss (iron chelation will affect multiple cellular processes with unknown consequences in vivo). Given that EMBO Reports is primarily aimed at molecular and cellular biology, perhaps the manuscript would benefit from some reorganization and trimming down on the non-essential in vivo data. Below are some relatively minor points to help improve the manuscript:

1) The authors should change the title, as they have not specifically shown that mitophagy supresses HCC - they have only shown that iron chelation supresses it. To do this would require specific inhibition of mitophagy in the mouse models and I'm not sure how the authors would do this (nor do I suggest they try here).

2) I recommend that the authors start their manuscript with the molecular studies and then include just the key in vivo data at the end - showing iron chelation increases mitophagy and this correlates with reduced HCC. I think this could make the manuscript more approachable to the general readership of EMBOR.

2) Related to monitoring mitophagy in vivo, the authors should specify how they define a mitophagosome-like structure. Indeed, data based on the quantification of EM images are very uncertain. Figure 1D, the central image showed probably the endoplasmic reticulum surrounding endosomes/mitochondria. At the left, the structures considered as mitophagosome-like structure seems to be too small and I cannot see mitochondria inside them.

3) I was a little confused with the in vivo data showing that DFP treatment improved mitochondrial function (Figure S1G); yet later in cells, DFP treatment inhibits mitochondrial function. How do the authors reconcile this?

4) Results based on immunofluorescence staining are sometimes not convincing. Authors could add a zoom of each individual channel to better appreciate the colocalization.

5) Given that siRNA has notorious off-target effects, and this is the first evidence for FTMT playing a role in mitophagy, can the authors provide controls for this? For example, in Figure 5 could they include a rescue experiment with siRNA-resistant FTMT?

6) In Figure 5A, the quantification of mitophagy by FACS after DFP treatment shows a high ratio signal of around 40%. But in Figure 2A-C, in the same cell line, this value is around 15%. Why? Also, perhaps Fig. 2A could be better annotated to show excitation wavelength, rather than pH?

7) In Figure 6D, are cells treated with DFP or is the overexpression of FTMT and NCOA4 enough to drive the interaction/colocalization?

8) In general, titles of sections are not consistent with conclusions made at the end of the paragraph.

Referee #2:

This work shows that mitophagy triggered by the iron chelator deferiprone (DFP) requires induction of mitochondrial ferritin (FTMT), which interacts with the autophagic cargo receptor NCOA4 in the outer membrane of depolarized mitochondria. Evidence is provided that this mechanism inhibits development of hepatocellular carcinoma (HCC) in mouse models of NASH-related HCC, while FTMT staining in human NASH biopsies imply a role of this protein in liver disease progression.

The overall findings are novel. The experiments are appropriately designed and the data are of high technical quality. There are, however, issues that require clarification.

1) Was DFP administered daily to the mice? Did this treatment cause anemia? Hematological data should be shown.

2) While the FTMT siRNA data in Fig. 5 demonstrate the involvement of FTMT in DFP-mediated suppression of tumor growth and are supportive to the author's model, it should be noted that there is a substantial literature on (experimental) iron chelation therapy against cancer. The concept is based on inhibitory effects of iron chelation on cell cycle and DNA synthesis via ribonucleotide reductase. How do these previous findings fit with the data herein?

3) In a related issue, the authors discuss on p. 22 that FTMT can retain more iron than cytosolic ferritin (ref 32). This has also been shown in a tumor xenograft model of FTMT (PMID 16757684); the data may be relevant to this work. Is it possible that iron retention in FTMT contributes to HCC inhibition?

4) Fig. 1E shows that DFP is more potent inducer of autophagy/mitophagy compared to the other chelators DFO and DFX. A major difference between these drugs is that DFP can remove iron from mitochondria, while the others cannot. Is mitochondrial iron chelation essential for FTMT induction? In other words, is MTFT induction a response to mitochondrial or cytosolic iron deficiency or both? Can DFO and DFX induce FTMT in cells and inhibit HCC in mice?

5) The DFP dose of 1 mM is shown to damage mitochondria (Fig. S6B). Is mitochondrial damage

necessary for FTMT induction?

6) The finding that FTMT is transcriptionally induced by iron chelation is novel and it was previously thought that FTMT is not iron-regulated. Nevertheless, this appears to be a late response that requires long incubations with DFP (24 h). A time course would be informative.

Minor
1) The description of the grading of mitophagosome-like structures (p. 19) is confusing.

2) On p. 22, please note that only the H- subunit of cytosolic ferritin has ferroxidase activity.

Referee #3:

Dr. Keisuke Hino group and colleagues presented a research article entitled 'Iron loss-induced mitophagy mediated by mitochondrial ferritin suppresses hepatocellular carcinoma'. In combination of both mouse and cell culture studies show that iron chelator deferiprone induces mitochondrial ferritin (FTMT), leading to the generation of the damaged mitochondria-pre-FTMT-NCOA4-LC3-II complex, and finally results in mitophagic degredation of damaged mitochondria. The project was very well performed with sufficient data convincingly showing the importance of elimination of damaged mitochondrial in cancer prevention.

Addressing the below questions will increase the quality of the paper
1. Since mitophagy is a key to understand the mechanisms of this paper, it would be nice the authors could give a succinct summary of the mechanisms of mitophagy and its roles in health and ageing (PMIDs: 30154567; 31375365; 31416937)
2. Fig. 1D, the DFP 0.075 mg/g group, the EM data of mitophagy was not convincing.
3. Fig. 2F, the Bafinomycin group for some gels was missing
4. Fig. 3B,D, etc. It is not to label the exact weigh of the proteins, we need to label the protein ladder-related sizes around: eg., TFR, the 100 kDa band of protein ladder was just upper of TFR (indicating the protein size of TFR was just less than 100 Kda, matching with the proposed size of 98 kDa).
5. Are there any other mechanisms of DFP-induced mitophagy? Can DFP induce mitochondrial fission? Can it induce the expression of other mitophagy-related proteins, like FUNDC1, NIX, BNIP3, BCL2L13 etc?

Response to referees

Dear Editor,

We thank the Editor and the reviewers for their careful reading of the manuscript and appreciate their constructive comments. We have now performed additional experiments and have revised the manuscript to address each of concerns raised by reviewers. We also have added the super-resolution images of immunofluorescence staining to show the localization of FTMT, LC3, NCOA4 and Tom20, using a single molecule microscopy, HM-1000 (Figure 3D and Figure 4D). Revised sentences are shown in dark red color in the revised manuscript.
Because we have revised the manuscript by starting with molecular studies of iron loss-induced mitophagy that were followed by *in vivo* data, the orders of figures in original manuscript has been largely changed in the revised manuscript. Please refer to a correspondence table of figure orders in the original manuscript and those in the revised manuscript described below.

Order of Figures in original manuscript	Order of Figures in revised manuscript
Figure 1A	Figure 6C
Figure 1B	Figure 6D
Figure 1C	Figure 6E
Figure 1D	Substituted with Figure 6F
Figure 1E	Figure 1A
Figure 2A	Figure 1B
Figure 2B	Figure 1C
Figure 2C	Figure 1D
Figure 2D	Figure 1E
Figure 2E	Figure 1F
Figure 2F	Figure 1G
	Figure 2A (newly added)
Figure 3A	Figure 2B
Figure 3B	Figure 2C

Figure 3C	Figure 2D
	Figure 2E (newly added)
Figure 3D	Figure 7A
Figure 4A	Figure 2F
Figure 4B	Figure 2G
Figure 4C	Figure 2H
Figure 4D	Figure 2I
Figure 4E	Figure 2J
Figure 4F	Figure 2K
Figure 5A	Figure 3A
Figure 5B	Figure 3B
	Figure 3C (newly added)
	Figure 3D (newly added)
Figure 5C	Figure 7B
Figure 5D	Figure 7C
Figure 5E	Figure 7D
Figure 5F	Figure 7E
Figure 6A	Figure 4A
Figure 6B	Figure 4B
Figure 6C	Figure 4C
	Figure 4D (newly added)
Figure 6D	Figure 4E
Figure 6E	Figure 4F
Figure 6F	Figure 4G
Figure 7A	Figure 5A
Figure 7B	Figure 5B
Figure 7C	Figure 5C
Figure 7D	Figure 5D
Figure 7E	Figure 5E
Figure 7F	Figure 5F
Figure 8F	Figure 6A
Figure 8B	Figure 6B

Figure 8C	Deleted
Figure 8D	Deleted
Figure 8E	Deleted
Figure 8F	Figure 8
Figure S1A	Figure EV4A
	Figure EV4B (newly added)
Figure S1B	Figure EV4C
Figure S1C	Figure EV4D
Figure S1D	Figure EV4E
Figure S1E	Figure EV4F
Figure S1F	Figure EV4G
Figure S1G	Figure EV4H
Figure S1H	Deleted
Figure S1I	Figure EV5A
Figure S1J	Figure EV5B
Figure S1K	Figure EV1A
Figure S2A	Figure EV1B
Figure S2B	Figure EV1C
Figure S3A	Figure EV1D
	Figure EV1E (newly added)
Figure S3B	Figure EV1F
Figure S3C	Figure EV5C
Figure S3D	Figure EV1G
Figure S4A	Figure EV2A
Figure S4B	Figure EV5D
Figure S4C	Figure EV5E
Figure S4D	Figure EV5F
Figure S5A	Figure EV2B
Figure S5B	Figure EV2C
Figure S5C	Figure EV2E
Figure S5D	Figure EV2D
Figure S5E	Figure EV2F

Figure S5F	Figure EV2G
Figure S6A	Figure EV3A
Figure S6B	Figure EV3B
Figure S6C	Figure EV3C
Figure S6D	Figure EV3D
Figure S7A	Deleted
Figure S7B	Deleted

Referee #1:

1) The authors should change the title, as they have not specifically shown that mitophagy suppresses HCC - they have only shown that iron chelation suppresses it. To do this would require specific inhibition of mitophagy in the mouse models and I'm not sure how the authors would do this (nor do I suggest they try here).

We thank the reviewer for the constructive suggestion. According to the reviewer's suggestion we have changed the title as shown below, focusing on the molecular mechanisms behind iron loss-induced mitophagy.
The title is "*Iron loss triggers mitophagy through induction of mitochondrial ferritin.*"

2) I recommend that the authors start their manuscript with the molecular studies and then include just the key in vivo data at the end - showing iron chelation increases mitophagy and this correlates with reduced HCC. I think this could make the manuscript more approachable to the general readership of EMBOR.

We thank the reviewer for the constructive suggestion. According to the reviewer's comments, we have revised the manuscript by starting with molecular studies of iron loss-induced mitophagy that were followed by *in vivo* data. For this reason, we have also revised the Introduction and deleted the data of mitochondrial ferritin expression in human liver biopsy specimens.

3) Related to monitoring mitophagy in vivo, the authors should specify how they define a mitophagosome-like structure. Indeed, data based on the quantification of EM images

are very uncertain. Figure 1D, the central image showed probably the endoplasmic reticulum surrounding endosomes/mitochondria. At the left, the structures considered as mitophagosome-like structure seems to be too small and I cannot see mitochondria inside them.

We appreciate the reviewer's critical comments. We defined a mitophagosome-like structure as a spherical structure with an isolation membrane containing high electron density cellular components with a membranous structure (lines 94-96 on page 6). According to the reviewer's comments, we have presented EM images which was originally shown in Figure 1D as Figure 6F in revised manuscript to show clearer mitophagosome-like structures. In Figure 6F, the swollen mitochondria with morphological abnormality are observed in the central image in the absence of DFP, while the fragmented mitochondria surrounded by isolation membrane are observed in the presence of DFP in the right image.

4) I was a little confused with the in vivo data showing that DFP treatment improved mitochondrial function (Figure S1G); yet later in cells, DFP treatment inhibits mitochondrial function. How do the authors reconcile this?

We apologize to you for the confusing description. To address the comments by the reviewer, we added the following sentences in the section of Results (lines 361-365 in page 18). "*Although these results are seemingly contradictory to the reduced mitochondria function by DFP in vitro (Figures 5A, 5B, EV3A, and EV3B), it appears to be reasonable that clearance of damaged mitochondria by DFP-induced mitophagy for a certain period of time (12 or 26 weeks in vivo) lead to the improvement of mitochondrial function in vivo.*"

5) Results based on immunofluorescence staining are sometimes not convincing. Authors could add a zoom of each individual channel to better appreciate the colocalization.

We thank the reviewer for constructive and critical comments. According to the reviewer's comment, we have added a zoom picture of each immunofluorescence

staining (Figure 3B, Figures 4C and 4E, Figures 6A and 6B, Figures EV2C, EV2E, EV2F and EV2G, and Figure EV3C).
We have also added the super resolution images of some immunofluorescence staining using the single-molecule microscopy that enabled the detection of aggregated proteins in spatial resolution of 23-40 nm (Figure 3D and Figure 4D). These super-resolution images were helpful for confirming the colocalization of LC3 and overexpressed FTMT at the mitochondria and the colocalization of endogenous NCOA4 and FTMT at the mitochondria.

6) Given that siRNA has notorious off-target effects, and this is the first evidence for FTMT playing a role in mitophagy, can the authors provide controls for this? For example, in Figure 5 could they include a rescue experiment with siRNA-resistant FTMT?

We thank the reviewer for the constructive suggestion. According to the reviewer's comments, we monitored the rescue of mitophagy using siRNA-resistant FTMT. The results of the rescue experiments are shown in Figures 3C and 3D. We also added the description of these data in the section of Results (lines 175 - 187 on page 10).

7) In Figure 5A, the quantification of mitophagy by FACS after DFP treatment shows a high ratio signal of around 40%. But in Figure 2A-C, in the same cell line, this value is around 15%. Why? Also, perhaps Fig. 2A could be better annotated to show excitation wavelength, rather than pH?

We appreciate the reviewer's comments. The difference of the mitophagy quantification is explained by the difference of DFP dose between two experiments (10 μM in Figure 1D and 1 mM in Figure 3A). In Figure 1F, 10 μM (=0.01 mM) of DFP showed a high ratio signal of around 15%, and 1 mM of DFP showed a high ratio signal of around 35%. To explain this, we added the description in the section of Results (lines 169-173 on page 9). We could not compare the effects of iron chelators on mitophagy induction at concentration more than 10 μM due to cytotoxic effect of DFX.
According to the reviewer's comment, we showed excitation wavelength instead of pH in Figure 1B.

8) In Figure 6D, are cells treated with DFP or is the overexpression of FTMT and NCOA4 enough to drive the interaction/colocalization?

It is exactly as you said. Cells treated with DFP or those with FTMT overexpression were enough to drive the interaction of FTMT with NCOA4. However, mutagenesis at the specific residues (R82 in FTMT and I489 and W497 in NCOA4) abrogated this interaction between these two proteins. We added this explanation in the section of Results (line 215 on page 11 – line 227 on page 12).

9) In general, titles of sections are not consistent with conclusions made at the end of the paragraph.

We thank the reviewer for the constructive suggestion. According to the reviewer's comment, we changed the titles of sections (lines 87-88 on page 6, lines 118-119 on page 7, lines 193-194 on page 10, line 259 on page 13, line 280 on page 14, line 319 on page 16, and line 333 on page 17).

Referee #2:

1) Was DFP administered daily to the mice? Did this treatment cause anemia? Hematological data should be shown.

We thank the reviewer for the constructive suggestion. DFP was administered daily to the mice. According to the reviewer's comment, we measured hemoglobin levels before and after treatment with DFP in STAM mice and DMBA + HFD mice. The results are shown in Figure EV4B. We also added the description about these results in the section of Results (lines 345-346 on page 17). Thus, DFP treatment did not cause anemia.

2) While the FTMT siRNA data in Fig. 5 demonstrate the involvement of FTMT in DFP-mediated suppression of tumor growth and are supportive to the author's model, it should be noted that there is a substantial literature on (experimental) iron chelation therapy against cancer. The concept is based on inhibitory effects of iron chelation on

cell cycle and DNA synthesis via ribonucleotide reductase. How do these previous findings fit with the data herein?

We thank the reviewer for the critical comments. According to the reviewer's comment, we cited a literature (reference #46; Shi *et al. Cell Mol Life Sci* **72**: 983-997, 2005) that reported the downregulation of cell proliferation by FTMT, and discussed a role of FTMT in anticancer effects of iron chelators. We added the description about this speculation in the section of Discussion (lines 447-454 on page 23).

3) In a related issue, the authors discuss on p. 22 that FTMT can retain more iron than cytosolic ferritin (ref 32). This has also been shown in a tumor xenograft model of FTMT (PMID 16757684); the data may be relevant to this work. Is it possible that iron retention in FTMT contributes to HCC inhibition?

We thank the reviewer for drawing our attention to this important reference. We found that the extent of decrease in iron content was greater in the cytoplasm than in mitochondria upon FTMT induction by DFP, even though these were *in vitro* data. These results seem to be relevant to the previous report that FTMT over expression inhibits xenograft tumors in nude mice via cytosolic iron deprivation, and raise a possibility that FTMT induction may suppress tumor development by affecting tumor iron homeostasis via shunting iron into mitochondria. We have added this discussion in the section of Discussion (lines 454-459 on page 23).

4) Fig. 1E shows that DFP is more potent inducer of autophagy/mitophagy compared to the other chelators DFO and DFX. A major difference between these drugs is that DFP can remove iron from mitochondria, while the others cannot. Is mitochondrial iron chelation essential for FTMT induction? In other words, is FTFT induction a response to mitochondrial or cytosolic iron deficiency or both? Can DFO and DFX induce FTMT in cells and inhibit HCC in mice?

We thank the reviewer for the critical comments. We measured cytoplasmic and mitochondrial iron content in Huh7 cells treated with DFP, DFX or DFO, and found that DFP chelated iron from the mitochondria but DFX and DFO did not (Figure 2A).

Additionally, we confirmed that treatment with DFX or DFO did not result in increased expression of FTMT in the same dose as DFP (Figure EV1E). These results suggested that mitochondrial iron deficiency may be required for FTMT induction apart from the stabilization of HIF1α-SP1 axis. Consequently, we added the 2 figures (Figure 2A and Figure EV1E) and the description in the section of Results (lines 121-123 on page 7 and line 129 on page 7 – line 130 on page 8) and in the section of Discussion (lines 455-459 on page 23). Antitumor effect of DFO against xenograft liver tumor in nude mice has been reported (Cancer 1992: 70; 2051-6). We are going to investigate whether FTMT plays a role in anticancer effect of DFO in mice in near future.

5) The DFP dose of 1 mM is shown to damage mitochondria (Fig. S6B). Is mitochondrial damage necessary for FTMT induction?

We thank the reviewer for the critical comments. DFP dose (0.01 mM) that did not damage the mitochondria (Figure EV3B) increased the expression of FTMT (Figure 2C). These results suggested that mitochondrial damage may not be necessarily required for FTMT induction. We have added this discussion in the section of Discussion (lines 430-434 on page 22).

6) The finding that FTMT is transcriptionally induced by iron chelation is novel and it was previously thought that FTMT is not iron-regulated. Nevertheless, this appears to be a late response that requires long incubations with DFP (24 h). A time course would be informative.

We thank the reviewer for the critical comments. According to the reviewer's comment, we measured *FTMT* mRNA levels before and 3, 6, 12, and 24 hours after DFP treatment in Huh7 cells (Figure 2E). As a result, DFP-induced transcriptional regulation of FTMT was a late response that requires incubation with DFP at least for 24 hours. On the other hand, the expression of HIF1α has been reported to increase following 2h-treatment with DFP (Cell Stress 2020: 5; 99-113). This contradiction raised a possibility that there exists another factor other than HIF1α-SP1 signaling for DFP-regulated FTMT induction. We added these results and discussion in the sections of Results (lines137-140 on page 8) and Discussion (lines 427-430 on page 22).

Minor

1) The description of the grading of mitophagosome-like structures (p. 19) is confusing.

As we were advised by the Editor and the Referee 1 to reorganize the manuscript, focusing on the *in vitro* molecular study and trimming down the non-essential *in vivo* data, we have deleted the data of FTMT expression in human liver biopsy specimens from the manuscript.

2) On p. 22, please note that only the H- subunit of cytosolic ferritin has ferroxidase activity.

According to the reviewer's comment, we revised the description in the section of Discussion (line 408-409 on page 21).

Referee #3:

1. Since mitophagy is a key to understand the mechanisms of this paper, it would be nice the authors could give a succinct summary of the mechanisms of mitophagy and its roles in health and ageing (PMIDs: 30154567; 31375365; 31416937)

We thank the reviewer for drawing our attention to these important references. As we reorganized the manuscript according to the comments by the Editor and the Referee 1, we largely changed the Instructions, focusing on the mechanisms of mitophagy and its role in ageing and diseases with citation of the references you could suggest (References #2, 3 and 7).

2. Fig. 1D, the DFP 0.075 mg/g group, the EM data of mitophagy was not convincing.

We thank the reviewer for a careful assessment of the figures. According to the reviewer's comment, we have presented the EM image which was originally shown in Figure 1D as Figure 6F in revised manuscript to show clearer mitophagosome-like structures.

3. Fig. 2F, the Bafinomycin group for some gels was missing.

We thank the reviewer for a careful assessment of the figures. According to the reviewer's comment, we have reexamined the immunoblotting with including the Bafilomycin group (Figure 1G and Figure EV1C).

4. Fig. 3B, D, etc. It is not to label the exact weigh of the proteins, we need to label the protein ladder-related sizes around: eg., TFR, the 100 kDa band of protein ladder was just upper of TFR (indicating the protein size of TFR was just less than 100 Kda, matching with the proposed size of 98 kDa).

We thank the reviewer for a careful assessment of the figures. According to the reviewer's comment, we have labelled the protein ladder-related sizes for all immunoblotting images (Figures 1A, 1G, 2C, 2I, 2J, 2K, 3A, 3C, 4A, 4B, 4F, 4G, 5E, 5F, and 7A, and Figures EV1C, EV1D, EV1E, EV3D, EV5A, EV5B, EV5C, and EV5D).

5. Are there any other mechanisms of DFP-induced mitophagy? Can DFP induce mitochondrial fission? Can it induce the expression of other mitophagy-related proteins, like FUNDC1, NIX, BNIP3, BCL2L13 etc?

We thank the reviewer for the critical comments. Interestingly, we found that DFP enhanced the expression of dynamin-related protein 1 (DRP1), a regulator for mitochondrial fission and induced the expression of NIX but not FUNDC1, as shown below. Moreover, a recent study reported that NIX and BNIP3 increased in human neuroblastoma cells upon DFP treatment (Zhao et al. *Cell Stress*, **4**:99-113; 2020. Based on these observations, it can be speculated that iron chelation with DFP induce mitochondrial fission. However, we did not include these results in the manuscript, since it was difficult to fit these results to the manuscript flow focusing on the mechanisms behind iron loss-induced mitophagy. Unfortunately, we could not assess the expression of BNIP3 and/or BCL2L13 because of the delayed delivery of antibodies to these proteins due to COVID 19.
(Figure for referees not Shown.)

1st Revision - Editorial Decision 21st Jul 2020

Dear Prof. Hino

Thank you for submitting the revised version of your manuscript. It has now been seen by all of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• All articles published beginning 1 July 2020, the EMBO Reports reference style changed to the Harvard style for all article types. Details and examples are provided at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat Please update the reference style accordingly.
• Please provide source data as one file per figure.
• We noted that there are some mismatches between the provided source data and figure panels (Figure 2C, 2I). Moreover, source data of EV Fig. 3D was mislabeled as 3C.
• We realized that the primer sequences were provided as a separate file. Please include them in the manuscript, in the Materials&Methods section.
• Papers published in EMBO Reports include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the key findings of the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb.
• We note that the synopsis image provided is too detailed for it to be visible when resized to 550x400 pixels (which will be its final size when published online). Please provide a simplified synopsis image.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

I'm happy to sat that the authors have adequately addressed my concerns.

Referee #2:

The revised manuscript is improved and all major issues have been addressed

Referee #3:

The authors had performed extensive experiments and revised the manuscript accordingly, leading to a great improvement of the manuscript. This reviewer was pleased of the effort by the authors in the COVID-1 crisis.

Dear Editor Tiebe

Many thanks for your positive and kind response.

I have revised the manuscript, the synopsis, and the synopsis image according to your advice as described below. I have uploaded the revised manuscript, all figures, and the source data through manuscript central. As the formatted revised manuscript is not activated with track changes, please confirm the attached word document with track changes activated.

I sincerely hope that our manuscript is accepted for publication in the EMBO reports.

Best regards,

Keisuke Hino

2nd Revision - Editorial Decision 12th Aug 2020

Prof. Keisuke Hino
Kawasaki Medical School
Hepatology and Pancreatology
577
Matsushima
Kurashiki, Okayama 7010192
Japan

Dear Dr. Hino,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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Corresponding Author Name: Keisuke Hino, Atsushi Tanaka
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-50202V2

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 iustified
- 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 χ2 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?	We referred to the similar experiments in the references and our previous experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We referred to our previous similar experiments.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA. Samples or animals that were not excluded.
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.	No special steps were taken. We allocated animals/samples to no treatment group and treatment group. In some cases two doses of iron chelators were allocated in treatment group.
For animal studies, include a statement about randomization even if no randomization was used.	We included the following statement: Four-week-old mice were orally administered with 0.0375 mg/g body weight of DFP, 0.075 mg/g body weight of DFP or distilled water for 12 weeks for STAM mice and for 26 weeks for DMBA + HFD mice.
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.	We did group allocation without subjective bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, they are justified as appropriate.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used statistical methods as appropriate.
Is there an estimate of variation within each group of data?	No, there is not.

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

http://grants.nih.gov/grants/olaw/olaw.htm
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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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Is the variance similar between the groups that are being statistically compared?	Yes, it is.

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).	NA. We used antibodies that are all commercially available.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	A NASH-related HCC mouse model (STAM mouse, SMC Laboratories, Inc, Tokyo, Japan) was developed as described previously [38]. Briefly, 2-day-old male C57BL/6J (Clea, Tokyo, Japan) mice were injected with streptozotocin (200 µg/mouse) and fed a high-fat diet (HFD-32, Clea) from the age of 4 weeks, which was followed by the development of NASH and HCC at 8 and 18 weeks of age, respectively. We also used DMBA-treated mice fed an HFD as an obesity-related HCC mouse model [39]. Briefly, 4-5-day-old male C57BL/6J mice were administered 50 µl of 0.5% DMBA (chemical carcinogen) solution to their dorsal surface and fed an HFD from the age of 4 weeks, which was followed by the development of HCC until 30 weeks of age. The mice were bred and maintained according to the guidelines approved by the Institutional Animal Care Use Committee (Kawasaki Medical School).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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 confirmed compliance.

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.	NA