EMBO reports

The mitochondrial Ca2+ uptake regulator, MICU1 is involved in cold stress-induced ferroptosis

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Dear Dr. Ichijo,

Thank you for submitting your manuscript to EMBO Reports. Three referees agreed to review your manuscript. So far, we have received two referee reports that are copied below. Given that both referees are in fair agreement that you should be given a chance to revise the manuscript, I would like to ask you to begin revising your study along the lines suggested by the referees.

Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. As soon as we receive the final report on your manuscript, we will forward it to you as well.

Referees express interest in the analysis. However, they also raise important concerns that need to be addressed to consider publication here.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript. As the reports are below, and I think all points need to be addressed, I will not detail them here.

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.

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2. Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases.

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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 (<http://embor.embopress.org/authorguide>). 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 (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

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:

<http://embor.embopress.org/authorguide#expandedview>.

- 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 <http://embor.embopress.org/authorguide#sourcedata>.

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 <http://embor.embopress.org/authorguide#datacitation>.

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 <http://embor.embopress.org/authorguide#dataavailability>).

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:

Nakamura, et al present an interesting follow-up to their recent finding that cold stress, which is a major obstacle for organ transplantation, can induce lipid peroxidation and ferroptosis. Using a CRISPR-based knockout screen, the authors identified the mitochondrial calcium uptake regulator MICU1 as a ferroptosis regulator. MICU1 enhances mitochondrial calcium influx, sustaining mitochondrial matrix potential (MMP) and causing the accumulation of lipid peroxides. Overall, the authors present strong evidence for their argument. The manuscript could be improved with the following changes:

Major points

1. Suppression of MMP hyperpolarization also inhibits cystine deprivation-induced ferroptosis. Does cystine starvation or erastin treatment respond to loss of MICU1? Similarly, While Dixon et al. reported no effect of BAPTA-AM on erastin treatment in their system (HT1080 cells), it would be worthwhile to confirm whether this is the case in A549 cells. If this is the case, the authors may want to use erastin to circumvent some of the experimental issues described (line 121-3, 153-4) caused by the low temperature requirement.

2. In lines 138-143, the authors suggest that complete loss of MCU, as opposed to loss of its activator MICU1, can activate alternative pathways to increase the mitochondrial calcium pool. The authors generated MCU knockouts in an MICU1-deficient cell line, restoring cell death. Are mitochondrial calcium pools suppressed in this scenario?

3. MICU1 and MICU2 are both regulatory subunits of MCU, and play opposing roles in regulating mitochondrial calcium uptake. If MICU1-dependent calcium uptake enhances ferroptosis, can MICU2 overexpression suppress ferroptosis through the same mechanism? This additional experiment may enhance the broader claim that MCU-dependent calcium influx regulates cold stress-induced ferroptosis.

4. The epistasis of the proposed pathway is confusing. While it is clear that mitochondrial calcium influx drives MMP hyperpolarization, according to Figures EV3G and H, both the uncoupler and ETC inhibitor inhibited mitochondrial calcium influx. The authors acknowledge this confusion in the text, but it would be very helpful to sort out this paradoxical finding.

Minor points

Graphs measuring relative calcium concentration or MMP are confusing - points are connected across different cell types, but why these points are connected is unclear. If these are showing each replicate, then the control should be normalized to 1 in order to make the graphs clearer.

Referee #3:

In this work, the authors studied the molecular underpinnings how cold stress triggers ferroptosis. While most of the studies performed so far in the ferroptosis field have used pharmacological or genetic models to induce ferroptosis, this study uses cold stress to induce ferroptosis in a more "pathophysiological" way. Using a genome-wide CRISPR/Cas screen the authors identified MICU1, a regulator the mitochondrial Ca2+ uptake, as an important player in cold stress-induced ferroptosis in the lung cancer cell line A549 besides the classical players of ferroptosis such as ACSL4. Targeted deletion of MICU1 in A549 cells protects against lipid peroxidation, mitochondrial Ca2+ uptake, mitochondrial membrane depolarization and associated oxidative stress. A further mapping of the different domains of MICU1 found that the DIME interaction and dimerization domains are essential for the pro-ferroptotic effects by MICU1. The findings are novel and interesting and the data is presented in a straightforward manner. Moreover, in light of the importance of cold stress-induced cell loss and tissue detriment as for instance occurring during organ transplantation, the findings might be relevant for certain pathophysiological contexts. As such, I just have just a few minor comments:

- Since all the studies were performed with one cell line, one may wonder how general these cell-protective effects of MICU1 ablation might be. At least, the authors should discuss this. Moreover, since this mechanisms proposed is evidently relevant for organ transplantation, the authors should elaborate on the expression profile of MICU1 in related organs.
- CRISPR/Cas mediated knockout of MICU1 causes resistance to cold stress-mediated ferroptosis. Would it be protective also against chemical inducers of ferroptosis such as erastin and RSL3?
- P4: The last paragraph doesn't read well and needs to be rephrased.
- P5: The authors mention they measured Fe2+ with a ferrous iron specific probe but do not provide any data. This set of data should be included in the supplementary information.
- Fig. 3g: How would untargeted decylubiquinone behave in cold stress induced ferroptosis as compared to MitoQ (see e.g. Friedmann Angeli et al NCA 2014)?
- Finally, the link between mitochondrial Ca2+ in the matrix and its impact on the generation of lipid ROS needs to be more thoroughly discussed. This is intriguing as the main ferroptosis regulator GPX4 is not present in the mitochondrial matrix arguing for a lipid ROS signal in this subcellular compartment.

Response to the reviewers:

In the revised manuscript, all modifications are highlighted in red.

Referee #1:

Nakamura, et al present an interesting follow-up to their recent finding that cold stress, which is a major obstacle for organ transplantation, can induce lipid peroxidation and ferroptosis. Using a CRISPR-based knockout screen, the authors identified the mitochondrial calcium uptake regulator MICU1 as a ferroptosis regulator. MICU1 enhances mitochondrial calcium influx, sustaining mitochondrial matrix potential (MMP) and causing the accumulation of lipid peroxides. Overall, the authors present strong evidence for their argument. The manuscript could be improved with the following changes:

We thank the reviewer's constructive comments. Based on his/her comments, we employed several experiments as mentioned.

Major points

1. Suppression of MMP hyperpolarization also inhibits cystine deprivation-induced ferroptosis. Does cystine starvation or erastin treatment respond to loss of MICU1? Similarly, While Dixon et al. reported no effect of BAPTA-AM on erastin treatment in their system (HT1080 cells), it would be worthwhile to confirm whether this is the case in A549 cells. If this is the case, the authors may want to use erastin to circumvent some of the experimental issues described (line 121-3, 153-4) caused by the low temperature requirement.

We thank the reviewer's suggestion. We have analyzed the involvement of MICU1 and cytosolic Ca^{2+} chelation in erastin-induced ferroptosis, but both perturbations did not suppress cell death. These findings suggest that the involvement of MICU1 and cytosolic Ca^{2+} may be selective for cold stress-induced ferroptosis. (Fig. EV. 5A-C). Thus, unfortunately, we could not use erastin to circumvent the experimental issues. Please see line 235-239.

2. In lines 138-143, the authors suggest that complete loss of MCU, as opposed to loss of its activator MICU1, can activate alternative pathways to increase the mitochondrial calcium pool. The authors generated MCU knockouts in an MICU1-deficient cell line, restoring cell death. Are mitochondrial calcium pools suppressed in this scenario?

We thank the reviewer's comment. Considering cell death was restored in MICU1 and MCU double deficient cells, mitochondrial calcium may increase under cold stress via alternative pathways. Due to some experimental limitations in which we could only observe the quite small differences of mitochondrial calcium in MICU1 KO cells, it is difficult to address the effect of MCU knockout in MICU1 deficient cells, but we added some sentences to discuss this potential scenario. Please see line 155-156.

3. MICU1 and MICU2 are both regulatory subunits of MCU and play opposing roles in regulating mitochondrial calcium uptake. If MICU1-dependent calcium uptake enhances ferroptosis, can MICU2 overexpression suppress ferroptosis through the same mechanism? This additional experiment may enhance the broader claim that MCU-dependent calcium influx regulates cold stress-induced ferroptosis.

We are grateful for the reviewer's constructive suggestions. We have analyzed the effect of MICU2 knockdown on cell death, instead of overexpression of MICU2. However, MICU2 deficiency by siRNAs did not promote cell death in relative early time point, i.e., 16 hours after cold stress (Fig. EV. 2K and L). Thus, it seems that MICU2 is not involved in this cell death. Please see line 169-175.

4. The epistasis of the proposed pathway is confusing. While it is clear that mitochondrial calcium influx drives MMP hyperpolarization, according to Figures EV3G and H, both the uncoupler and ETC inhibitor inhibited mitochondrial calcium influx. The authors acknowledge this confusion in the text, but it would be very helpful to sort out this paradoxical finding.

We apologize for this confusing explanation. We added and rephrased some sentences to figure out the mutual relationship between MMP and mitochondrial Ca^{2+} regulation. Please see line 200-201.

Minor points

Graphs measuring relative calcium concentration or MMP are confusing - points are connected across different cell types, but why these points are connected is unclear. If these are showing each replicate, then the control should be normalized to 1 in order to make the graphs clearer.

We apologize for these confusing figures and thank the reviewer's suggestion. We remade the figures (Fig. 3D, 3F, EV. 3B, E, H, J, L) in which the controls were normalized to 1. For Ca^{2+} measurement experiments, to compare different cells or inhibitors, all data were normalized by the control conditions (WT or DMSO) at the end points of the line graphs, i.e., at the time of 60 min after cold stress of control conditions. For MMP measurement experiments, to compare different cells, all data were normalized by the WT at the end points of the line graphs, i.e., at the time of 5 h after cold stress of control conditions, subtracted from the WT after FCCP treatment. For MMP measurement experiments of inhibitors, all data were normalized by DMSO at the end points of the line graphs. Please see the Method section for details.

Referee #2

In this study, the authors examined cold-stress induced ferroptosis, and performed a genome-wide CRISPR screen to identify proteins involved, and discovered MICU1, a key regulator of the activity of MCU, the major mechanism for mitochondrial Ca uptake. Different types of measurements were made to attempt to identify the role of the phenotype observed, namely that knockdown of MICU1 promoted resistance to the hallmark features of ferroptosis. However, the logical is often confusing, and many experiments lack sufficient methodological details and controls. Additional experiments seem peripheral to the main story, such as attempting to identify a cold sensor, and examining the effect of electron transport chain inhibitors. In aggregate, the conclusions are not clear.

We thank the reviewer's constructive comments. Based on his/her comments, we employed several experiments as mentioned below.
Related to the latter parts, we apologize if the reviewer felt our story was less connected. However, our aims of this research were focusing on whole cell death mechanisms and cellular signaling under cold stress. Besides, the role of ETC and Ca^{2+} channel in ferroptosis was not fully understood and still controversial. We believe that these findings would be important for the field of ferroptosis and cold stress signaling. Thus, we investigated these players in cold stress-induced ferroptosis.

1. Line 118, it is concluded that the MICU1 mutants that did not rescue cold stress-induced lipid peroxidation and death was due to a failure to activate MCU. However , this conclusion cannot be correct. If it was the case, then the EF hand mutant, which cannot activate MCU, should not have been able to rescue, but it did. The data are instead consistent with the idea that gatekeeping, i.e. keeping MCU permeability turned off, is necessary for cold-stress-induced death, as suggested in line 113-114. This is further supported by the presence of cold-stress induced death with MCU knocked down. And further supported by the abrogation of the MICU1 effect by knockdown of MCU, without the need to invoke other mitochondrial Ca uptake pathways. Since lack of MICU1 promotes a mitochondrial Ca leak, the data suggest that a Ca2+ leak promotes survival, i.e. resistance to cold stress-induced death. An alternate hypothesis is that MICU1 has MCU-independent functions as

suggested in a recent study

We apologize that the sentence of our conclusion is misleading. We admit this reviewer's comment, and on the light of the mutation analysis, we deleted the concluding sentence: "Since ΔDID (K438A;R440A;R443A) and ΔDIMER (C463A) mutations are located in the C-terminus (Fig 2A), it is likely that these mutants lack MICU1-MCU interactions necessary for complex formation, resulting in failure to activate MCU.", and alternatively discussed the role of gatekeeping but not activating function of MICU1on MCU as follows: "ΔDID (K438A;R440A;R443A) and ΔDIMER (C463A) mutations are located in the C-terminus (Fig 2A) and lack MICU1-MCU/MICU2 interactions necessary for gatekeeping MCU complex. On the other hand, EF hand mutation of MICU1 impairs its Ca^{2+}-dependent activating but not gatekeeping activity of MCU. One of the interpretations of these data would be that the ability of MICU1 to interact with MCU and/or MICU2 and to serve as a gatekeeper for MCU may be necessary for cold stress-induced ferroptosis". Please see line 118-124. We also agree with the alternative hypothesis about the MCU-independent MICU1's function, based on the results of MCU knockdown and knockout experiments. We thus added sentences for this possibility. Please see line 151 and 154.

2. The mitochondrial [Ca] measurements are problematical. Rhod-2 localizes not only to mito, but to cytoplasm as well. The authors add 200 uM MnCl2 to the medium to quench cytoplasmic Rhod-2, but there is no demonstration here regarding its effectiveness, so how much of the signal is cyto and mito is unclear. The problem is exacerbated in the MICU1 KO cells because MCU, lacking MICU1 gatekeeping, is Mn permeable in these cells, which will quench the mito Rhod-2 signal. Without a calibration mechanism, differences in WT vs MICU1 KO are quite difficult to interpret. Finally, the authors suggest that the data indicate that mito [Ca] is lower with MICU1 KO, but that is clearly not the case in other studies that have shown, including in patient cells, that lack of MICU1 results in constitutively elevated mito [Ca]. The conclusion in lines 155-156 therefore seems unwarranted.

Considering the reviewer's comment, we totally agree with comments on Mn^{2+}. Indeed, supplementation of Mn^{2+} during measuring has some problems, but without Mn^{2+}, signal intensity keeps increasing even under room temperature (in the appendix figure 3A (left)). As the previous paper mentioned (PMID: 24212091), adding Mn^{2+} worked better to solve this problem during measuring mitochondrial Ca^{2+}. That's why we used Mn^{2+}.
Surely, other papers show that MICU1 KO have an opposite effect to our data on mitochondrial Ca^{2+} (PMID: 23101630), but all of them demonstrate that MICU1 KO cells become more sensitive to cell death, which is also completely different from our observed phenotype. Besides, some papers show that MICU1 KO could suppress mitochondrial Ca^{2+} uptake in some stimuli (PMID: 20693986, 23747253). Thus, we are considering that these differences depend on stimuli and/or cell lines.
In addition, considering the possibility that MICU1 KO constitutively elevated mitochondrial Ca^{2+} from cytosol and this elevation may be the cause of inhibition of cell death under cold stress, we investigated the effect of MCU overexpression on cell death, and MCU overexpressing cells did not suppress cold stress-induced cell death (Fig. EV. 2H and I).
Together with the data of cytosolic Ca^{2+}, MICU1 KO may reduce mitochondrial Ca^{2+} influx in a cold stress-specific manner. However, since we agree with having many problems with measuring Ca^{2+} in our experiments, we deleted the concluding sentence: "Altogether, we concluded that MICU1 deficiency decreased mitochondrial Ca^{2+} uptake in the presence of MCU under cold stress, leading to the suppression of cold stress-induced cell death." . Alternatively, we added some sentences describing not Ca^{2} concentration, but speculations related to MICU1's gatekeeping or MCU-independent functions: "Altogether, the gatekeeping activity and/or the potential MCU-independent functions of MICU1 may be important for cold stress -induced ferroptosis." Please see line 174-175.

3. What is the evidence for the conclusion in lines 192-194, since MICU1 manipulations were not part of these studies. Rather, the results in this paragraph seem to recapitulate previous data.

We apologize for recapitulating previous data. We deleted these sentences.

4. No calibration control for membrane potential measurements, i.e. normalizing to cccp.

We thank the reviewer's suggestion. We performed the experiments again with calibration by the FCCP treatment at the end of measurement (For detail, please see Methods and Protocols). We could obtain almost the same data as previous ones (Fig. 3B and D) and found that MICU1 KO suppressed MMP hyperpolarization after cold stress.

5. Some key details are missing. There are no details regarding the antibodies used. Unclear is how the cold stress was maintained during the fluorescence reads for calcium and membrane potential. Presumably the cells warned back up to room temperature?

We apologize for complexity of the procedure details. We have uploaded the datasheet of reagents and antibodies as the reagent table, so we would appreciate it if you would check the reagent table. Regarding cold stress condition, we set the inner temperature of the microplate reader at the minimum; 10.2 ˚C during the measurement. For reviewer's concern of rewarming in measurement, we enclosed an appendix figure 3A (right) which shows the actual temperature. Although the temperature slightly increased during measurement, it did not reach room temperature. Please see the methods section for details.

6. The degree of knockdown with siRNAs is remarkable (expanded fig 2a).

We thank the reviewer's note.

7. Fig 2B.. how could MICU1 dimers been seen in reducing conditions?

We apologize for this misleading figure. We used DTT as the reducing reagents, and some proteins are resistant to DTT to be reduced at all disulfide bands compared to other reducing agents such as β-mercaptoethanol (PMID: 15741328). Thus, we suppose these bands may be the MICU1's dimers. But, as we did not confirm these bands are derived from dimer, we indicated these bands as potential DTT-resistant dimer on the immunoblot figure (Figure 2B).

Referee #3:

In this work, the authors studied the molecular underpinnings how cold stress triggers ferroptosis. While most of the studies performed so far in the ferroptosis field have used pharmacological or genetic models to induce ferroptosis, this study uses cold stress to induce ferroptosis in a more "pathophysiological" way. Using a genome-wide CRISPR/Cas screen the authors identified MICU1, a regulator the mitochondrial Ca2+ uptake, as an important player in cold stress-induced ferroptosis in the lung cancer cell line A549 besides the classical players of ferroptosis such as ACSL4. Targeted deletion of MICU1 in A549 cells protects against lipid peroxidation, mitochondrial Ca2+ uptake, mitochondrial membrane depolarization and associated oxidative stress. A further mapping of the different domains of MICU1 found that the DIME interaction and dimerization domains are essential for the pro-ferroptotic effects by MICU1. The findings are novel and interesting and the data is presented in a straightforward manner. Moreover, in light of the importance of cold stress-induced cell loss and tissue detriment as for instance occurring during organ transplantation, the findings might be relevant for certain pathophysiological contexts. As such, I just have just a few minor comments:

We thank the reviewer's constructive comments. Based on his/her comments, we employed several experiments using other cell lines and other stimuli as mentioned below.

- Since all the studies were performed with one cell line, one may wonder how general these cell-protective effects of MICU1 ablation might be. At least, the authors should discuss this. Moreover,

since this mechanisms proposed is evidently relevant for organ transplantation, the authors should elaborate on the expression profile of MICU1 in related organs.

We thank the reviewer's suggestion. We have analyzed the involvement of MICU1 in cold stress-induced ferroptosis in kidney and liver cell lines (HEK293A and HepG2) to emphasize the cytoprotective effect of MICU1inhibition for organ transplantation. Then, we found that MICU1 KD suppressed cell death dramatically in both cell lines (Fig. EV. 5F-I).
We added the figure to show the expression levels of MICU1 in human normal tissues using ReFex database. It shows that MICU1 expresses ubiquitously in human organs and MICU1 can be one of the promising targets for organ preservation (Fig. EV. 5E). Please see line 260-264.

- CRISPR/Cas mediated knockout of MICU1 causes resistance to cold stress-mediated ferroptosis. Would it be protective also against chemical inducers of ferroptosis such as erastin and RSL3?

We are grateful to the reviewer's constructive suggestions. We used A549 and HT-1080 cells to investigate whether MICU1 is involved in other stimuli-induced and MMP mediated ferroptosis. However, erastin-induced ferroptosis were not suppressed by MICU1 KO (Fig.EV. 5B and C). Therefore, these results suggest that MICU1 is selectively required for cold stress-induced ferroptosis. Please see line 237-239.

- P4: The last paragraph doesn't read well and needs to be rephrased.

We apologize that there are some undistinguishable parts in our initial manuscript.
We rephrase it. Please see line 149-156.

- P5: The authors mention they measured Fe2+ with a ferrous iron specific probe but do not provide any data. This set of data should be included in the supplementary information.

We apologize that the data is missing in our initial manuscript.
We enclosed that data in appendix figure 3B.

- Fig. 3g: How would untargeted decylubiquinone behave in cold stress induced ferroptosis as compared to MitoQ (see e.g. Friedmann Angeli et al NCA 2014)?

We thank the reviewer's constructive suggestions. We have analyzed the involvement of mitoQ analog; unspecific localized antioxidant (decyl ubiquinone) in cold stress-induced ferroptosis. As another paper shows, decyl ubiquinone protected cell death more efficiently than mitoQ (Fig. 3J). This would suggest that most parts of ROS in cold stress-induced ferroptosis are from mitochondria, but other ROS source may also exit to induce this cell death. Please see line 218-224.

- Finally, the link between mitochondrial Ca2+ in the matrix and its impact on the generation of lipid ROS needs to be more thoroughly discussed. This is intriguing as the main ferroptosis regulator GPX4 is not present in the mitochondrial matrix arguing for a lipid ROS signal in this subcellular compartment.

We are thankful to reviewer's suggestions. We added the discussion about the link between lipid ROS and Ca^{2+} influx to matrix and subsequent signal transduction. Please see line 213-218.

1st Revision - Editorial Decision 3rd Feb 2021

Dear Dr. Ichijo,

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

I apologize for the delay in getting back to you. It took longer than anticipated to receive the referee reports due to the recent holiday season.

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:

• Please rename the 'Data and code availability' section as 'Data Availability'. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>), and the accession numbers and database should be listed in a formal "Data Availability " section - if it is not applicable, a statement should be made as follows: 'no data were deposited in a public database'.
• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• Please upload figures as individual files.
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Fig EV2K+L callouts are missing.
Fig EV3G+H callouts are missing.
Fig EV5 callouts are missing.
• We note the following about dataset EV legends: The legends are missing. Table EV1 should be renamed as Dataset EV1, Table EV2 should be renamed as Table EV1.
• We note that Table EV3 is the results of statistical tests, which can also be seen in the figures. If this is the case, please remove the Table EV3, making sure that all information is indeed in the figures/legends.
• The source data provided for Fig EV5 G and H is mislabeled as Fig EV5 H.
• Please move the 'reagent table' to the Materials & Methods section of the manuscript, and update the callouts in the text.
• Please split the source data into one file per figure.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both 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 paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• 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. I am aware that the comments were made on an earlier version of the manuscript, please use the attached document as a reference and perform the changes on the

latest version of the text.

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:

the authors have addressed all questions from this reviewer.

Referee #3:

The authors have appropriately addressed all my concerns. As such, I have no further comments.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 15th Feb 2021

Dear Dr. Ichijo,

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: Hidenori Ichijo
Journal Submitted to: Report
Manuscript Number: EMBOR-2020-51532V2

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 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?	No statistical mechods were used to determine sample size in advance.
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.	NA
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	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, we described precisely in the methods section and figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	No statistical methods were used to assess it.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes, we provided tha data as means +/- SEM.
Is the variance similar between the groups that are being statistically compared?	Yes, we hypothesized that the variance is similar between the groups. Otherwise, we mentioned this as paired samples is in the methods section.

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).	We have provided these information in the reagents and tools table.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We have provided these information in the reagents and tools table and methods section.

* 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.	NA
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.	NA

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
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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.	We do not anticipate that our study fall under dual use research restrictions.