EMBO reports

Interferon-mediated repression of miR-324-5p potentiates necroptosis to facilitate antiviral defense

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DOI: 10.15252/embr.202154438

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Review Timeline:

Submission Date:	7th Dec 21
Editorial Decision:	4th Jan 22
Revision Received:	19th Apr 22
Editorial Decision:	20th May 22
Revision Received:	23rd May 22
Accepted:	1st Jun 22

Editor: Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **4th Jan 2022**

Dear Prof. He,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your 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 of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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 file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

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

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

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 & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets 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. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) 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://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments were performed, if these were biological or technical replicates, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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10) Please remove the highlights section from the title page.

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.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

Review, 23rd December 2021

Title: Interferon-mediated repression of miR--p potentiates necroptosis to facilitate antiviral defense
Manuscript # EMBOR202154438V1

General Remarks:
This study describes a mir that targets MLKL to repress MLKL translation and which is inhibited by STAT1. Upon interferon signalling, which activates STAT1, this can result in a well known increase in MLKL levels. The authors propose that the levels of this mir therefore determine sensitivity of cells to necroptosis.

The main problems are that:
1. the effects are subtle. This is a perennial problem with mirs but it does impose a greater level of proof to be confident in interpretation. One suggestion is to include ifna/b in more of the assays (Fig. 5onwards) as this provides truly independent biological experiments.
2. The authors attribute all interferon induced upregulation of MLKL to this mir. Since by their own admission this mir regulation is not present in mouse etc, but MLKL gets upregulated by ifns in mouse cells, it is extremely hard to believe that this mir is the sole regulator of ifn induced MLKL. Furthermore why should all species upregulate MLKL but only a subset utilise the mir? This is a significant practical and theoretical weakness. It could be addressed by doing CHIP assays of the MLKL promoter (to look for STAT1 binding to MLKL promoter) and run-on assays.
3. missing proof that the identified mir binding site in MLKL is necessary for mir regulation.
4. The relevance of the IAV experiments given that IAV induces apoptosis, just as much if not more so necroptosis.

Specific Remarks:

Note: I make these as I read through the manuscript and synthesize them into a coherent criticism above. Some times I think they need to be specifically addressed, some times they are just comments. However I reproduce them here so that they might help you understand where I find difficulties etc.

Fig. 2A needs an MLKL alone control.
Line 169 - It is stretching the meaning of the word great to call this a great reduction in pMLKL.
Line 175 - difference in MLKL level in MKN45 is slight
Line 206 - what causes the large difference in size of the exogenous MLKL? It should be described in text and/or figure legend.
Line 247, several much earlier papers than the ones cited showed MLKL upregulation by interferons - e.g. 2013 10.1073/pnas.1301218110 or 2017 10.1038/s41418-017-0031-1 . Since both of these examples are in mouse cells, which do not apparently have this mir binding site, it raises the question why have a conserved pathway of MLKL upregulation (ifns) via a different mechanism?
Line 263 - knocking down STAT1 abolishes IFNγ induced down regulation it does not restore it.
Fig. 5F-G and I-J needs corresponding Western blots, among others showing that ruxolitinib stops STAT1 phosphorylation.
Fig. 6B-D needs corresponding Western blots.
The Fig. 6F experiment should be done with IFNγ rather than TNF, with corresponding Western blots as this tests the hypothesis that IFNγ inhibits the mir. Given the small differences seen I personally would suggest that the authors also look at ifna/b signalling as they show that these also upregulate MLKL and it would bolster confidence in the results. Especially since IFNß is the most upregulated by IAV infection.
Line 285 - IAV PR8 induces apoptosis and necroptosis, Nogusa et al 2016, in fact my reading of this paper is that loss of FADD provides more protection than loss of MLKL (which provides none, Fig. 4A), same for Kuriakose et al. Therefore the authors should check whether the mir affects IAV induced apoptosis (ie experiments without Z-VAD-FMK)
Since Kuriakose et al showed that ZBP is interferon inducible during IAV infection the authors should check that it is not affected by the mir.

target(s) is misspelled on a number of occasions in the manuscript.

Referee #2:

The manuscript by Dou et al. describes the role of miR-324-5p as a regulator of necroptosis in human cells in vitro. miR-324-5p targets the 3' UTR of MLKL leading to its downregulation. MLKL itself is a key effector protein of necroptosis as, once necroptosis is triggered, MLKL gets phosphorylated by RIPK3, oligomerizes and translocates to the cell membrane in order to disrupt membrane integrity. The authors utilized a miRNA screening approach to find new regulators of necroptosis in HT-29 cells and miR-324-5p was among the strongest inhibitors of necroptosis. While RIPK1 and RIPK3 are not affected by miR-324-5p, the protein levels of MLKL and p-MLKL are significantly reduced after miRNA transfection. Furthermore, the authors show that miR-324-5p targets the 3' UTR of MLKL independent of the seed region. Treatment of cells with type I or II interferons, downregulates the levels of miR-324-5p which results in increased levels of MLKL and thus increases the cell's sensitivity for necroptosis. Finally, Dou et al. show that expression of miR-324-5p enhances replication of influenza A virus in human PBMCs and HT-29. Consequently, loss of miR-324-5p inhibits IAV replication, as it increases MLKL expression and eliminates the

infected cells.
Overall, the manuscript is well-written, timely and provides inside into a necroptosis-regulating miRNA. New mechanisms of cell death regulation are very important for the biology of pathogenic infections but also for autoimmunity and cancer. Although most of the figures are clear, some questions remain that need to be addressed before the manuscript can be considered for publication in EMBOR. Thus, I ask the author to reply to the following major and minor comments:

Major Comments:

1. Major information on the PBMCs is missing - source? Ethics statement? Healthy donors? Fresh blood? How isolated? How were Macrophages generated? Etc.
2. CRISPR-Cas9-generated cells: miR-324 is located in the intron of gene ACADVL. Does the genomic deletion of miR-324 affect ACADVL levels? Please check.
3. All Figures: Please specify the exact number of replicates for each figure (do not just write 'at least 3 replicates'.).
4. All Figures: What does 'representative data are shown' for the bar graphs mean? Does this mean that only the data of 1 replicate is shown? If so, please show the summarized data for replicates and only use a representative image for the WB data.
5. Fig 5A/C: Why does LPS/polyIC treatment not upregulate MLKL levels as both stimuli induce type I IFN and activate the JAK/STAT pathway? Does treatment of LPS or polyIC affect the levels of miR-324-5p? Please add this data.
6. Fig 7B: Please add a control gene for the ruxolitinib treatment. An ISG such as CXCL10 or Viperin could be used.
7. IAV experiments: Please add Western Blot data showing how IAV infection affects protein levels and phosphorylation of MLKL.
8. The paper by Kumar et al (2018, Journal of Virology) which is also referenced in this manuscript shows that miR-324-5p regulates type I IFN. Did the authors made the same observation? Is the miR-324-dependent modulation of IAV replication based on the regulation of type IFN or MLKL or an effect of both? Please perform additional experiments to further discriminate between both effects. In addition, an experiment using miR-324-5p in combination with blocking IFN/JAK-STAT pathway would help to get more insight into the role of miR-324-5p in modulation of IAV-replication.
9. How does type I IFN affect the levels of MLKL in the miR-324-KO cells? Is the regulation of MLKL through IFN fully dependent or the miRNA or is it only a minor effect? Please add this experiment to the manuscript.

Minor Comments:
1. Fig 1B: please mark the NC control in the graph
2. What about the miR-324-5p levels in HT-29? Is it present at basal state and does T+S+Z treatment affect the miR levels?
3. Fig 1D: Do all three treatments reduce MLKL expression in HeLa endogenous MLKL cells? Please add this data as this is a necessary control experiment.
4. Graphs that show the expression of miR-324-5p show the y-axis title "relative miR-324-5p mRNA expression" -> miR-324-5p is not a messenger RNA. Please change to miRNA or remove mRNA.
5. Methods: antibody dilutions are missing
6. Methods: Western blot -> What blocking buffer? Secondary antibody?
7. Methods: miRNA screening -> miRNA and lipofectamine concentrations are missing
8. Fig 7H: Why are single data points shown here but not for the other experiments?
9. PBMC data: Are replicates for experiments with PBMC-derived cells based on different donors?

Referee #3:

Interferon mediated repression of miR-324-5p potentiates necroptosis to facilitate antiviral defense.
Duo et al.

Summary:
Authors identify miR324-5p as a specific mediator of necroptosis in by screening a library of candidate miRNAs. The show that miR324-5p is downregulated upon treatment with interferons, and this downregulation results in the increased expression of MLKL to enhance the execution of necroptosis in response to multiple stimuli. This regulatory mechanism appears to be species specific, as human and primate MLKL are sensitive to miR324-5p regulation, while murine cells are not. Finally, they demonstrate that miR324-p5 regulation of MLKL may play a role in influenza virus induced necroptosis and virus control.

Reviewer Impressions:
Manuscript details an interesting and important aspect of necroptosis control. The authors demonstrate by multiple lines of investigation that in human and primate cells, miR324-5p is an interferon repressed gene that regulates the expression of MLKL, and this regulation potentiates the ability of treated or infected cells to undergo necroptosis. To this reviewers knowledge, there are a number of peer-reviewed studies showing miRNA regulation of necroptosis, including some that show targeting of MLKL (e.g. FASEB J . 2019 Mar;33(3):3523-3535.). However, the authors of this manuscript provide important and interesting insight into the cellular mode of regulating miR324-5p via interferons, which is consistent with the physiological role that interferon is reported to play in necroptosis. The article is well written (although needs a little copyediting, please see below for examples), and appears to address the known literature adequately, although a little more time and care could be spent discussing miRNA

regulation of necroptosis and how this study augments what is known. As such, this reviewer believes the findings presented here are of general interest to the community and is suited for consideration in EMBOReports.

Major concerns:
One of the major points of the paper is that the regulation of MLKL by miR324-5p is species specific, with primate and human cells being responsive and murine cells not. While the presentation of the 3'UTR sequences is interesting, and the functional data presented in fig 4 is compelling. Indeed the non-seed sequences identified are necessary for regulation. However, the author's conclusions would be better supported by data showing that the introduction of critical sequences into the 3'UTR of murine or porcine cells is sufficient to potentiate miR324-5p regulation. This could be done using the reporter assay system presented in figure 3. Additionally, is miR324-5p conserved in those species? Is it downregulated by interferon treatment?

Authors show that miR324-5p is regulated by interferons. It was a little surprising that they continued their characterization with type II interferon, but finished their manuscript with a viral infection well known for its interactions with the type I interferon system. While their choice of inhibitors and siRNA likely had pan-IFN effects, their signaling mechanism are distinct. Does repression of miR324-5p involve IRFs or just STATs? It would have been expected, at least by this reviewer, that their more detailed analysis used IFNalpha and/or beta. A few additional experiments (specifically figs 5E and H) with IFNbeta treatment would add support to the authors conclusions.

Minor Concerns:
Typo in line 792 - need a space between "analysisof"
Figure EV4, panel B figure legend - panel does not show the expression of MLKL.

Referee #1:

General Remarks:

This study describes a mir that targets MLKL to repress MLKL translation and which is inhibited by STAT1. Upon interferon signalling, which activates STAT1, this can result in a well known increase in MLKL levels. The authors propose that the levels of this mir therefore determine sensitivity of cells to necroptosis.

The main problems are that:

1. the effects are subtle. This is a perennial problem with mirs but it does impose a greater level of proof to be confident in interpretation. One suggestion is to include ifna/b in more of the assays (Fig. 5onwards) as this provides truly independent biological experiments.

Response:

We appreciate these comments. Based on the reviewer's suggestion, we have examined the effect of IFN-β treatment on miR-324-5p and added the data to Fig 5. As show in the revised Fig 5C and 5D, IFN-β stimulation increased MLKL expression and decreased miR-324-5p expression. Moreover, IFN-β-induced upregulation of MLKL expression was inhibited by miR-324-5p transfection. We have revised the text accordingly.

We further examined the effect of the JAK1/JAK2 inhibitor ruxolitinib on IFN-β-mediated downregulation of miR-324-5p. The addition of ruxolitinib blocked IFN-β-induced downregulation of miR-324-5p as well as IFN-β-induced upregulation of MLKL. Moreover, knockdown of STAT1 abolished IFN-β-induced downregulation of miR-324-5p and prevented IFN-β-induced upregulation of MLKL. We have added these results into Fig 5F and 5I, and revised the text accordingly.

2. The authors attribute all interferon induced upregulation of MLKL to this mir. Since by their own admission this mir regulation is not present in mouse etc, but MLKL gets upregulated by ifns in mouse cells, it is extremely hard to believe that this mir is the sole regulator of ifn induced MLKL. Furthermore why should all species upregulate MLKL but only a subset utilise the mir? This is a significant practical and theoretical weakness. It could be addressed by doing CHIP assays of the MLKL promoter (to look for STAT1 binding to MLKL promoter) and run-on assays.

Response:

We appreciate the reviewer's suggestions. The CHIP assay measuring STAT1 binding to the MLKL promoter has been performed in the mouse cells (Günther et al., J Clin Invest, 2016;126(11):4346-4360. PMID: 27756058). It has been shown that IFN-γ could directly induce the binding of STAT1 to the mouse *Mlkl* promoter region in mouse cells. This result suggests that IFNs could directly regulate *Mlkl* gene transcription via activation of STAT1 in some species such as mice. We have discussed this point raised by the reviewer in the revised manuscript. Our study indicates MiR-324-5p as a negative regulator of MLKL and necroptosis in higher mammals such as human beings and monkeys.

3. missing proof that the identified mir binding site in MLKL is necessary for mir regulation.

Response:

Based on the reviewer's suggestion, we synthesized a mutant form of miR-324-5p in which "AGG" in the predicted binding site was replaced by "UCC". We found that the mutant form of miR-324-5p failed to inhibit the luciferase activity of MLKL-3'UTR (A), supporting that the binding site "AGG" is required for miR-324-5p regulation. We have added this data as Fig 3C into the revised manuscript and revised the text accordingly.

Further, we generated a mutant form of murine MLKL-3'UTR by introducing the miR-324-5p binding region of human MLKL-3'UTR into the start site of mouse MLKL-3'UTR. We observed that this mutant form of mouse MLKL-3'UTR was negatively regulated by miR-324-5p, suggesting that introduction of these critical sequences is sufficient to potentiate the regulation of mouse MLKL-3'UTR by miR324-5p. We added this data as Fig 4F into the revised manuscript and revised the text accordingly.

4. The relevance of the IAV experiments given that IAV induces apoptosis, just as much if not more so necroptosis.

Response:

Thank you for pointing it out. It is known that IAV can induce apoptosis as well as necroptosis. We further examined the effect of miR-324-5p on IAV-induced apoptosis. We found that either transfection of miR-324-5p or siMLKL oligos did not affect the levels of cleaved caspase 3, suggesting that miR-324-5p does not affect IAV-induced apoptosis. We added this data as Fig EV5A into the revised manuscript and revised the text accordingly.

Specific Remarks:

Note: I make these as I read through the manuscript and synthesize them into a coherent criticism above. Some times I think they need to be specifically addressed, some times they are just comments. However I reproduce them here so that they might help you understand where I find difficulties etc.

Fig. 2A needs an MLKL alone control.

Response:

We have added the western blot analysis of MLKL into Fig 2A.

Line 169 - It is stretching the meaning of the word great to call this a great reduction in pMLKL.

Response:

We have revised the manuscript: “We observed that phosphorylation of MLKL was reduced in HT-29 cells transfected with miR-324-5p, while phosphorylation of RIPK1 and RIPK3 was not affected under the same conditions”.

Line 175 - difference in MLKL level in MKN45 is slight

Response:

We have performed the experiment again and confirmed that MLKL expression was downregulated by overexpression of miR-324-5p. This data was shown in Fig 2D in the revised manuscript.

Line 206 - what causes the large difference in size of the exogenous MLKL? It should be described in text and/or figure legend.

Response:

Thank the reviewer for the comment. In order to easily distinguish the exogenous and endogenous MLKL, we generated a DNA plasmid containing a HA and 3xFlag tags at the N terminal of MLKL. The exogenous MLKL is larger than the endogenous MLKL. We have revised the label in Fig 3D and described the tags in the method.

Line 247, several much earlier papers than the ones cited showed MLKL upregulation by interferons - e.g. 2013 10.1073/pnas.1301218110 or 2017 10.1038/s41418-017-0031-1 . Since both of these examples are in mouse cells, which do not apparently have this mir binding site, it raises the question why have a conserved pathway of MLKL upregulation (ifns) via a different mechanism?

Response:

Thank you for the comments. We have cited these papers in the revised manuscript. As discussed in Question 2, it has been reported that that IFN-γ could directly induce the binding of STAT1 to the mouse *Mlkl* promoter region in mouse cells (Günther et al., J Clin Invest, 2016;126(11):4346-4360. PMID: 27756058), suggesting that IFNs directly regulate *Mlkl* gene transcription via activation of STAT1 in mouse species. Of note, the mutant form of mouse MLKL-3'UTR, which was generated by introduction of the miR-324-5p binding region of human MLKL-3'UTR, could be negatively regulated by miR-324-5p. Therefore, our work demonstrates that the function of miR-324-5p in necroptosis has evolved in higher mammals, suggesting a species-specific mechanism for modulation of necroptosis. We have discussed this point in the discussion.

Line 263 - knocking down STAT1 abolishes IFNγ induced down regulation it does not restore it.

Response:

We have corrected this sentence: “Knocking down STAT1 abolished IFNs-induced downregulation of miR-324-5p and prevented IFNs-induced upregulation of MLKL (Fig 5H and 5I)”.

Fig. 5F-G and I-J needs corresponding Western blots, among others showing that ruxolitinib stops STAT1 phosphorylation.

Response:

Based on reviewer’s suggestion, we have added Western blot analysis of STAT1 phosphorylation. Addition of ruxolitinib abolished phosphorylation of STAT1 induced by IFN-β or IFN-γ. These results have been added into Fig 5F-5I in the revised manuscript. Please see the response to the Question 1.

Fig. 6B-D needs corresponding Western blots.

Response:

We have added corresponding Western blot analysis of MLKL into Fig 6B-D. Accordingly, the figure legends were also revised.

The Fig. 6F experiment should be done with IFNγ rather than TNF, with corresponding Western blots as this tests the hypothesis that IFNγ inhibits the mir. Given the small differences seen I personally would suggest that the authors also look at ifna/b signalling as they show that these also upregulate MLKL and it would bolster confidence in the results. Especially since IFNß is the most upregulated by IAV infection.

Response:

We examined cell viability induced by IFN-β and IFN-γ in HT-29 miR-324-5p$^{+/+}$ and miR-324-5p$^{-/-}$ cells. We found HT-29 miR-324-5p$^{-/-}$ cells were more sensitive to IFNs induced necroptosis and IFNs could induce higher expression level of MLKL in miR-324-5p$^{-/-}$ cells. We added these results as Fig 6G, 6H, and Fig EV4C into the revised manuscript and revised the text accordingly.

Line 285 - IAV PR8 induces apoptosis and necroptosis, Nogusa et al 2016, in fact my reading of this paper is that loss of FADD provides more protection than loss of MLKL (which provides none, Fig. 4A), same for Kuriakose et al. Therefore the authors should check whether the mir affects IAV induced apoptosis (ie experiments without Z-VAD-FMK).

Since Kuriakose et al showed that ZBP is interferon inducible during IAV infection the authors should check that it is not affected by the mir.

Response:

We examined the effect of miR-324-5p on apoptosis. Transfection of miR-324-5p did not affect caspase-3 activation post IAV infection. Please see the response to the

Question 4.

We also observed that transfection of miR-324-5p did not affect the expression level of ZBP1 (data were shown as below)[Figures for referees not shown.] .

target(s) is misspelled on a number of occasions in the manuscript.

Response:

The manuscript text has been revised following the reviewer's suggestion.

Referee #2:

The manuscript by Dou et al. describes the role of miR-324-5p as a regulator of necroptosis in human cells in vitro. miR-324-5p targets the 3' UTR of MLKL leading to its downregulation. MLKL itself is a key effector protein of necroptosis as, once necroptosis is triggered, MLKL gets phosphorylated by RIPK3, oligomerizes and translocates to the cell membrane in order to disrupt membrane integrity. The authors utilized a miRNA screening approach to find new regulators of necroptosis in HT-29 cells and miR-324-5p was among the strongest inhibitors of necroptosis. While RIPK1 and RIPK3 are not affected by miR-324-5p, the protein levels of MLKL and p-MLKL

are significantly reduced after miRNA transfection. Furthermore, the authors show that miR-324-5p targets the 3' UTR of MLKL independent of the seed region. Treatment of cells with type I or II interferons, downregulates the levels of miR-324-5p which results in increased levels of MLKL and thus increases the cell's sensitivity for necroptosis. Finally, Dou et al. show that expression of miR-324-5p enhances replication of influenza A virus in human PBMCs and HT-29. Consequently, loss of miR-324-5p inhibits IAV replication, as it increases MLKL expression and eliminates the infected cells.

Overall, the manuscript is well-written, timely and provides inside into a necroptosis-regulating miRNA. New mechanisms of cell death regulation are very important for the biology of pathogenic infections but also for autoimmunity and cancer. Although most of the figures are clear, some questions remain that need to be addressed before the manuscript can be considered for publication in EMBOR. Thus, I ask the author to reply to the following major and minor comments:

Major Comments:

1. Major information on the PBMCs is missing - source? Ethics statement? Healthy donors? Fresh blood? How isolated? How were Macrophages generated? Etc.

Response:

We appreciate the reviewer's questions. We described the Ethics statement in the revised manuscript. PBMCs were from whole blood from healthy donors. We isolated the PBMCs using Ficoll according to the manufacturer's instructions. Briefly, we diluted the blood with PBS containing 2% FBS, and layer the diluted cells on Ficoll reagent in a tube. Then, the tube was centrifuged at 400 x g for 30 minutes. The mononuclear cells at the interface would be harvested, and cultured in RPMI-1640 medium containing 10% FBS and 50 ng/ml MGSF. After 7 days, the macrophages were stimulated as indicated for further analysis. We have added the detail to the method.

2. CRISPR-Cas9-generated cells: miR-324 is located in the intron of gene ACADVL. Does the genomic deletion of miR-324 affect ACADVL levels? Please check.

Response:

Based on the reviewer's suggestion, we checked the expression level of ACADVL by qPCR. And we observed that knocking out of miR-324-5p did not affect ACADVL levels (data were shown as below)[Figures for referees not shown.] .

3. All Figures: Please specify the exact number of replicates for each figure (do not just write 'at least 3 replicates').

Response:

We have revised the manuscript according to the reviewer's suggestion.

4. All Figures: What does 'representative data are shown' for the bar graphs mean? Does this mean that only the data of 1 replicate is shown? If so, please show the summarized data for replicates and only use a representative image for the WB data.

Response:

Thank the reviewer for the suggestion. We have revised the method. The representative

data are shown of summarized data from two or three independent experiments, and one of them was used as a representative image.

5. Fig 5A/C: Why does LPS/polyIC treatment not upregulate MLKL levels as both stimuli induce type I IFN and activate the JAK/STAT pathway? Does treatment of LPS or polyIC affect the levels of miR-324-5p? Please add this data.

Response:

Based on the reviewer's suggestion, we have added the data of miR-324-5p levels after the treatment of LPS or Poly(I:C) into Fig 5C and revised the text accordingly. We found that the expression levels of MLKL and miR-324-5p were not significantly affected by LPS or Poly(I:C) treatment for 24h (Fig 5B and 5C in the revised manuscript), although the mRNA levels of type I IFNs (IFN-α and IFN-β) were slightly induced by LPS or Poly(I:C) at this time point (data were shown as below)[Figures for referees not shown.] . These results suggest that induction of type I IFNs by LPS or Poly(I:C) post 24h treatment may be not sufficient to affect the levels of miR-324-5p and MLKL.

6. Fig 7B: Please add a control gene for the ruxolitinib treatment. An ISG such as

CXCL10 or Viperin could be used.

Response:

Based on the reviewer's suggestion, we examined the CXCL10 level and found that IAV induced the expression CXCL10 and this induction was inhibited by the ruxolitinib treatment (data were shown as below)Poly(I:C) post 24h treatment . We also confirmed that ruxolitinib treatment blocked STAT1 phosphorylation (Fig 5F-G in the revised manuscript).

Fig 4 IAV induced expression of CXCL10 was inhibited by the ruxolitinib.

PBMC cells were incubated with 300 nM ruxolitinib for 2 h prior to IAV (H1N1 strain PR8) infection at MOI of 0.2 for 24 h. qPCR analysis for the expression of CXCL10.

7. IAV experiments: Please add Western Blot data showing how IAV infection affects protein levels and phosphorylation of MLKL.

Response:

Based on the reviewer's suggestion, we examined MLKL and p-MLKL levels post IAV treatment. IAV infection led to increased levels of MLKL and phosphorylated MLKL. We added these results as Fig EV5B into the revised manuscript and revised the text accordingly.

8. The paper by Kumar et al (2018, Journal of Virology) which is also referenced in this manuscript shows that miR-324-5p regulates type I IFN of IAV replication based on the

regulation of type I IFN or MLKL or an effect of both? Please perform additional experiments to further discriminate between both effects. In addition, an experiment using miR-324-5p in combination with blocking IFN/JAK-STAT pathway would help to get more insight into the role of miR-324-5p in modulation of IAV-replication.

Response:

According to the reviewer's suggestion, we examined IAV replication using miR-324-5p in combination with blocking IFN/JAK-STAT pathway. Ruxolitinb inhibited the replication of IAV and overexpression of miR-324-5p enhanced the IAV replication (data were shown as below)blocked STAT1 phosphorylation (Fig 5F-G in the revised manuscript).. These results shown that overexpression of miR-324-5p

can enhance IAV replication even in the presence of ruxolitinb. suggesting that miR-324-5p regulates IAV replication via modulating MLKL rather than type I IFN.

Fig 5 Overexpression of miR-324-5p can enhance IAV replication even in the presence of ruxolitinib.

PBMC-derived macrophages were treated with the 300nM ruxolitinib for 2 h prior to transfected with NC or miR-324-5p for 48h. qPCR analysis for the expression of M gene of IAV.

9. How does type I IFN affect the levels of MLKL in the miR-324-KO cells? Is the regulation of MLKL through IFN fully dependent or the miRNA or is it only a minor effect? Please add this experiment to the manuscript.

Response:

We examined cell viability induced by IFN-β and IFN-γ in HT-29 miR-324-5p$^{+/+}$ and miR-324-5p$^{-/-}$ cells. We found HT-29 miR-324-5p$^{-/-}$ cells were more sensitive to IFNs induced necroptosis and IFNs could induce higher expression level of MLKL in miR-324-5p$^{-/-}$ cells. We added these results as Fig 6G, 6H, and Fig EV4C into the revised manuscript and revised the text accordingly.

Minor Comments:

1.Fig 1B: please mark the NC control in the graph

Response:

We have added the NC control in the revised Fig 1B.

2.What about the miR-324-5p levels in HT-29? Is it present at basal state and does T+S+Z treatment affect the miR levels?

Response:

We examined MLKL and miR-324-5p levels in HT-29 and U937. We found that HT29 cells have similar expression level of miR-324-5p level compared to U937 cells (data were shown as below)[Figures for referees not shown.]. And we examined MLKL and miR-324-5p levels in HT-29 after the treatment of T+S+Z. We found mR-324-5p level was not affected by T+S+Z treatment (data were shown as below)[Figures for referees not shown.] .

Fig 6 HT29 cells have similar expression level of MLKL and miR-324-5p compared to U937 cells.

(A, B) qPCR analysis for the expression of MLKL (A) and miR-324-5p (B) in HT-29 and U937.

(C, D) qPCR analysis for the expression of MLKL (A) and miR-324-5p (B) in HT-29 that were treated with DMSO or T+S+Z for 4 h.

3.Fig 1D: Do all three treatments reduce MLKL expression in HeLa endogenous MLKL cells? Please add this data as this is a necessary control experiment.

Response:

We think this question may be related to Fig 3D which showed the data from HeLa endogenous MLKL cells. As shown in Fig 3D, the protein level of endogenous MLKL was reduced by all three transfections of miR-324-5p, siMLKL-3'UTR and siMLKL-CDS in HeLa cells. We added quantitative analysis of western blots. This data was added as Fig EV3C in the revised manuscript.

4.Graphs that show the expression of miR-324-5p show the y-axis title "relative miR-324-5p mRNA expression" -> miR-324-5p is not a messenger RNA. Please change to miRNA or remove mRNA.

Response:

We have revised the figures according to the reviewer’s suggestion.

5.Methods: antibody dilutions are missing

Response:

We have revised the method:

The following antibodies were used for western blot analysis: RIPK1 (BD Biosciences, 610458, 1:8000), RIPK3 (Prosci, 2283, 1:8000), human MLKL (Huabio, ET1601, 1:1000), mouse MLKL (Abgent, 142726, 1:2000), p-STAT1 (CST, 7649, 1:1000), STAT1 (CST, 9175, 1:1000), phospho-human MLKL (Abcam, 187091, 1:1000), Flag (Sigma, A8592, 1:10000), ZBP1(SantaCruz, 67258, 1:2000), caspase 3 (CST, 9662, 1:1000), cleaved caspase 3 (CST, 9664, 1:1000), and β-actin (Sigma, A2066, 1:20000).

6.Methods: Western blot -> What blocking buffer? Secondary antibody?

Response:

We have revised the method:

For Western blot analysis, cells were harvested and lysed in protein lysis buffer (20 mM Tris·HCl, 10% glycerol, 1% TritonX-100, 150 mM NaCl, 1 mM Na_3VO_4, 25 mM β-glycerol-phosphate, 0.1 mM PMSF) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor set (Roche). Cell lysates were fractionated on SDS-PAGE gels, and electrophoretically transferred to a NC membrane. The NC membranes were blocked in 5% non-fat Milk (PBS containing 0.1% Tween-20) for 1 h at room temperature. Membranes were incubated with indicated primary antibody for 2 h at room temperature. After washing to remove any unbound primary antibody, membranes were incubated with goat anti-Rabbit (LI-COR, 926-32211,1:10000) or goat anti-mouse (LI-COR, 926-32210,1:10000). Proteins were visualized using Odyssey Imaging system (LI-COR). Data were analyzed with ImageJ software. The band intensities of the analyzed proteins were normalized to that of β-actin. Three independent experiments were performed, and representative results are shown.

7.Methods: miRNA screening -> miRNA and lipofectamine concentrations are missing

Response:

We have revised the method:

The control non-target miRNA (negative control) and RIPK3 siRNA were used as the negative control and positive control, respectively. Human miRNAs (50 nM) were transferred into 96-well plates containing Lipofectamine 2000 (0.2 ul per well).

8.Fig 7H: Why are single data points shown here but not for the other experiments?

Response:

We have revised figures and present the individual data points.

9.PBMC data: Are replicates for experiments with PBMC-derived cells based on different donors?

Response:

We PBMCs were isolated from 5 healthy donors, and cells for replicates were from different donors.

Referee #3:

Interferon mediated repression of miR-324-5p potentiates necroptosis to facilitate antiviral defense.

Duo et al.

Summary:

Authors identify miR324-5p as a specific mediator of necroptosis in by screening a

library of candidate miRNAs. The show that miR324-5p is downregulated upon treatment with interferons, and this downregulation results in the increased expression of MLKL to enhance the execution of necroptosis in response to multiple stimuli. This regulatory mechanism appears to be species specific, as human and primate MLKL are sensitive to miR324-5p regulation, while murine cells are not. Finally, they demonstrate that miR324-p5 regulation of MLKL may play a role in influenza virus induced necroptosis and virus control.

Reviewer Impressions:

Manuscript details an interesting and important aspect of necroptosis control. The authors demonstrate by multiple lines of investigation that in human and primate cells, miR324-5p is an interferon repressed gene that regulates the expression of MLKL, and this regulation potentiates the ability of treated or infected cells to undergo necroptosis. To this reviewers knowledge, there are a number of peer-reviewed studies showing miRNA regulation of necroptosis, including some that show targeting of MLKL (e.g. FASEB J . 2019 Mar;33(3):3523-3535.). However, the authors of this manuscript provide important and interesting insight into the cellular mode of regulating miR324-5p via interferons, which is consistent with the physiological role that interferon is reported to play in necroptosis. The article is well written (although needs a little copyediting, please see below for examples), and appears to address the known literature adequately, although a little more time and care could be spent discussing miRNA regulation of necroptosis and how this study augments what is known. As such, this reviewer believes the findings presented here are of general interest to the community and is suited for consideration in EMBOReports.

Response:

We appreciate the reviewer's positive comments and valuable suggestions.

Major concerns:

One of the major points of the paper is that the regulation of MLKL by miR324-5p is species specific, with primate and human cells being responsive and murine cells not. While the presentation of the 3'UTR sequences is interesting, and the functional data presented in fig 4 is compelling. Indeed the non-seed sequences identified are necessary for regulation. However, the author's conclusions would be better supported by data showing that the introduction of critical sequences into the 3'UTR of murine or porcine cells is sufficient to potentiate miR324-5p regulation. This could be done using the reporter assay system presented in figure 3. Additionally, is miR324-5p conserved in those species? Is it downregulated by interferon treatment?

Response:

We appreciate these comments. Based on the reviewer's suggestion, we generated a mutant form of murine MLKL-3'UTR by introducing the miR-324-5p binding region "CTCTCATATCCTTCGGCATTG" of human MLKL-3'UTR into the start site of mouse MLKL-3'UTR. We observed that this mutant form of mouse MLKL-3'UTR was negatively regulated by miR-324-5p, suggesting that introduction of these critical sequences is sufficient to potentiate the regulation of mouse MLKL-3'UTR by miR324-5p. We added this data as Fig 4F into the revised manuscript and revised the text accordingly. Moreover, we synthesized a mutant form of miR-324-5p in which "AGG" in the predicted binding site was replaced by "UCC". We found that the mutant form of miR-324-5p failed to inhibit the luciferase activity of MLKL-3'UTR, supporting that the binding site "AGG" is required for miR-324-5p regulation. This result has been added as Fig 3C into the revised manuscript.

MiR-324-5p is conserved in human and mouse. IFNs induced the expression level of miR-324-5p in human cells, but had no obvious effect on miR-324-5p in mouse cells (data were shown as below)[Figures for referees not shown.]. These results suggest that miR-324-5p is not involved in the regulation of MLKL in mouse cells.

Authors show that miR324-5p is regulated by interferons. It was a little surprising that they continued their characterization with type II interferon, but finished their manuscript with a viral infection well known for its interactions with the type I interferon system. While their choice of inhibitors and siRNA likely had pan-IFN effects, their signaling mechanism are distinct. Does repression of miR324-5p involve IRFs or just STATs? It would have been expected, at least by this reviewer, that their more detailed analysis used IFNalpha and/or beta. A few additional experiments (specifically figs 5E and H) with IFNbeta treatment would add support to the authors conclusions.

Response:

We appreciate these comments. Based on the reviewer's suggestion, we have added IFN-β treatment into Fig 5. IFN-β stimulation increased MLKL expression and decreased miR-324-5p expression. Moreover, IFN-β-induced upregulation of MLKL

expression was inhibited by miR-324-5p transfection. The addition of ruxolitinib blocked IFN-β-induced downregulation of miR-324-5p as well as IFN-β-induced upregulation of MLKL. Moreover, knockdown of STAT1 abolished IFN-β-induced downregulation of miR-324-5p and prevented IFN-β-induced upregulation of MLKL. We have added these results into Fig 5F and 5H, and revised the text accordingly.

In addition, we found that knockdown of IRF1 did not affect the MLKL level induced

by IFN-β treatment (data were shown as below)[Figures for referees not shown.] .

Minor Concerns:

Typo in line 792 - need a space between "analysisof"

Response:

We have revised the manuscript accordingly.

Figure EV4, panel B figure legend - panel does not show the expression of MLKL

Response:

We have revised Figure EV4 and the figure legends.

Dear Prof. He,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports. Referee #1 has a remaining concern/suggestion I ask you to address in a final revised manuscript. Please also provide a response to the remaining point of referee #1.

Moreover, I have these editorial requests I also ask you to address:

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. Presently, many diagrams show only partial statistics.

- As the Western blots shown are significantly cropped, please provide the source data for the blots. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data for all the Western blots shown in the main and EV figures (scans of entire blots) together with the final revised manuscript. Please include size markers for the scans of entire blots, label the scans with figure and panel number, and send one PDF file per figure.

- Please add a formal 'Data Availability Section' after the Methods. This is now mandatory, like the conflict of interest statement. If no primary datasets have been deposited, please state this section (e.g. 'No primary datasets have been generated and deposited').

- In the first row of ethics section of the author checklist (row 91) you indicate that information on human participants, authority granting, and ethics approval could be found in the methods section of the manuscript. However, there is no such ethics statement in the manuscript text file. Moreover, it seems there is no data involving human participants in the study. Please check. Either change the box in the author checklist (to 'Not Applicable') or add the ethics statement to the methods section.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have done a good job in responding to my comments. However now they have clarified the tag on MLKL in Fig. 3 is an N-terminal HA and 3xFlag tag, I have an additional request/comment.

My request is that they acknowledge/reference the fact that for mouse MLKL an N-terminal FLAG tag renders MLKL inactive, which is obviously different to what the authors show here in Fig. 3G for human MLKL, see Hildebrand et al, 0.1073/pnas.1408987111 and Hildebrand et al, 10.1038/s41467-020-16819-z.

The comment is that if this were my work I would make doubly sure that the cell death measured in 3G is accurate before publishing. The murine data is strong and has been repeated by other groups (eg 10.1038/s41467-021-23474-5) and my group have unpublished data showing that N-terminal tagging affects human MLKL function too.

I note that whether N-terminal tagging affects MLKL function or not is not essential for the message of this figure and would be content with either outcome but it would be a shame to devalue the overall findings with a potentially silly oversight.

Referee #2:

The authors have sufficiently responded to all reviewers comments and the manuscript should be considered for publication in EMBOR.

Referee #3:

Summary (From previous review):
Authors identify miR324-5p as a specific mediator of necroptosis in by screening a library of candidate miRNAs. The show that miR324-5p is downregulated upon treatment with interferons, and this downregulation results in the increased expression of MLKL to enhance the execution of necroptosis in response to multiple stimuli. This regulatory mechanism appears to be species specific, as human and primate MLKL are sensitive to miR324-5p regulation, while murine cells are not. Finally, they demonstrate that miR324-p5 regulation of MLKL may play a role in influenza virus induced necroptosis and virus control.

Reviewer Impressions:
Manuscript details an interesting aspect of necroptosis control. The authors demonstrate by multiple lines of investigation that in human and primate cells, miR324-5p is an interferon repressed gene that regulates the expression of MLKL, and this regulation potentiates the ability of treated or infected cells to undergo necroptosis. Authors have taken significant steps to address reviewer comments from prior submission, which, in the opinion of this reviewer, has improved the manuscript. The findings presented are of general interest to communities studying cell death, innate immunity, and miRNA function. It is suitable for consideration for publication in EMBOReports.

Authors have adequately addressed prior reviewer critiques.

Dear Dr. Achim Breiling,

We are submitting our revised manuscript entitled “Interferon-mediated repression of miR-324-5p potentiates necroptosis to facilitate antiviral defense” (EMBOR-2021-54438V2) to ***EMBO Reports*** for consideration of publication. We appreciate all the suggestions from you and referee #1. We have reviewed the policy for competing interests. All authors declare that they have no competing interests. We have also corrected the subheading to 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section. We have clearly state figure legends (main and EV figures) as your suggestions. In addition, we have also submitted the source data for all the Western blots, and added a formal 'Data Availability' Section after the Methods. We have revised the author checklist (row 91). Primary human PBMCs isolated from whole blood were used in this study. The ethical statement for the use of PBMCs was shown in the methods section (highlighted).

The short summary of the findings and their significance:

IFNs activate the JAK/STAT1 pathway, resulting in the decrease of miR-324-5p and upregulation of MLKL, and thus potentiating IAV-infected cells necroptosis to limit viral replication.

Bullet points:

• MiR-324-5p downregulates MLKL and thus inhibits necroptosis

• MiR-324-5p targets MLKL in a seed region-independent and species-specific manner

• IFN-induced JAK-STAT1-mediated downregulation of miR-324-5p post-transcriptionally promotes MLKL expression

• The IFN-miR-324-5p-MLKL axis protects the host against IAV infection

A synopsis image with proper size and resolution has been submitted online.

Responses to Referee #1:

The authors have done a good job in responding to my comments. However now they have clarified the tag on MLKL in Fig. 3 is an N-terminal HA and 3xFlag tag, I have an additional request/comment.

My request is that they acknowledge/reference the fact that for mouse MLKL an

N-terminal FLAG tag renders MLKL inactive, which is obviously different to what the authors show here in Fig. 3G for human MLKL, see Hildebrand et al, 0.1073/pnas.1408987111 and Hildebrand et al, 10.1038/s41467-020-16819-z.

The comment is that if this were my work I would make doubly sure that the cell death measured in 3G is accurate before publishing. The murine data is strong and has been repeated by other groups (eg 10.1038/s41467-021-23474-5) and my group have unpublished data showing that N-terminal tagging affects human MLKL function too.

I note that whether N-terminal tagging affects MLKL function or not is not essential for the message of this figure and would be content with either outcome but it would be a shame to devalue the overall findings with a potentially silly oversight.

Response: We appreciate your evaluation and valuable comments for our manuscript. We have double-checked and confirmed that the tag was located at the C-terminal of human MLKL. Accordingly, we have revised it in the method section (highlighted).

We think that we have addressed all the concerns raised by you and Referee #1. Thank you very much for your consideration. We are looking forward to hearing from you.

Sincerely yours,

Sudan He, Ph.D.
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Dear Prof. He,

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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.

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Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

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Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods/Figures/Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure/Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure/Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	