EMBO reports

XAF1 prevents the hyperproduction of type I interferon caused by viral infection by targeting IRF7

Bao-qin Liu, Rong-bei Liu, Wen-ping Li, Xin-tao Mao, Yi-ning Li, Tao Huang, Hao-li Wang, Hao-tian Chen, Jiang-yan Zhong, Bing Yang, Renjie Chai, Qian Cao, Jin Jin, and Yi-yuan Li
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Dear Prof. Li,

Thank you for the submission of your research manuscript to EMBO reports. I 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, all referees 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 and in a 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 (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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

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When submitting your revised manuscript, we will require:

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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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3) 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

4) 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 in 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. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

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, 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 (also for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please also note our reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat
10) Please add scale bars of similar style and thickness to all the microscopic images (main and EV figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) 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.

12) Please have your revised manuscript carefully proofread by a native speaker.

Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript. Please find instructions on how to link the ORCID ID to the account in our manuscript tracking system in our Author guidelines: http://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

Moreover, we request that the corresponding author also provides an official institutional e-mail address!

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
Senior Editor
EMBO Reports

Referee #1:

In this study, Baoqin Liu et.al reported a negative feedback loop involved in IFN-I production, focusing on IRF7 protein stability. A diploma raised when macrophages faced infection of viruses: mRNA abundance of the strong inducer Irf7 remained elevated after infection, but the IRF7 protein did not increased in the same way. The authors found that protein level of IRF7 was strictly controlled by XAF1-XIAP-KLHL22 axis through ubiquitination-dependent proteasome degradation pathway. As an essential mechanism for cells to avoid overactive responses, this axis made an important role in regulation IFN-I production and authors regarded it as a novel target as therapeutic drugs for viral infection or chronic inflammatory diseases that IFN-I invovled.

Major points:
1. In fig1, authors identified the ARG1, which was an essential protein in M2 macrophage differentiation. Was there any difference in proportion of induced M2 macrophage between WT and XAF1 MKO?
2. When activated by viruses, IRF7 and IRF3 were phosphorylated and translocated to nuclear to promote transcription. It is known that phosphorylation of Serine 477/479 was essential for IRF7 activation (PMID:10893229), did the ubiquitination of IRF7 relied on its phosphorylation?
3. When referred to K48 ubiquitination, authors should confirm the result using K48 point mutation ubiquitin. Of note, FigS5D did not represent the lysine point mutation but a screen for candidates. The author should supplement the data.
4. The authors should improve the ubiquitination of IRF7 mediated by KLHL22 in vitro.

Minor points:
The entire writing languages of this work should be improved, and color/scale bar of some figures were wrong.
1.In the page5, line 113. "We next confirmed that KLHL22 (a substrate of BCR) is an E3 ligase that directly targets IRF7" may should be changed to "We next confirmed that KLHL22 (an adaptor of BCR) is an E3 ligase that directly targets IRF7", which is more accurate and more consistent with the abstract section.
2.In the page6, line 128. "we challenged wild-type (WT) bone marrow-derived macrophages (BMDMs) with Sendai virus (SeV)". The authors have already introduced the meaning of BMDM in the introduction section, so the abbreviation of BMDM can be used directly here.
3.There are some typos and grammar errors in the text. For example, In the page6, line 130. "post stimulation" should be separated by spaces.
4.In the page6, line 164. The abbreviation of "IFN-" should be unified.
In the page11, line 247. The abbreviation of "IRF7" should be unified.
In the page15, line 341. "KLHL22 is a substrate-specific adaptor of the BCR (BTB-CUL3-RBX1 (BCR) E3 ubiquitin ligase complex". The author expressed here repetition and cumbersomeness.
5.The labeling format of some figures is not uniform.
In the main Figure 7D. The minus sign should be changed to "-" instead of "_".
In the main Figure 7H. The abbreviations "K48-linked Ub" should be unified to "K48-Ub".
6.It is best to mark the full name of each domain in the main Figure 6K.
7.In the supplementary Figure 5C. The authors should label legend.
8.Some protein bands, especially "Actin" bands, are overexposed and look too strong and a bit ugly. It is recommended to control the exposure time and adjust the sample load to obtain high-quality results.
9.The format of the histogram is not uniform, and the thickness of the lines is different. It is recommended to have a uniform format.

Referee #2:

The article by Liu et al uses a combination of in vivo experiments and in vitro studies and infections to report several findings centred around IRF7 protein stability including that 1) XAF1 interacts with IRF7, promoting K48-linked ubiquitination and proteasomal degradation of IRF7 to restrict IFN-I induction 2) XIAP binds to IRF7 and inhibits IRF7 ubiquitination, promoting IFN-I induction and 3) KLHL22 conjugates K48-linked ubiquitin chains to IRF7 leading to its proteasomal degradation and a reduction of IFN-I. This work is potentially of great general interest as IRF7 is an interferon regulatory transcription factor of relevance to innate antiviral immunity and must be tightly regulated to avoid harming the host.

A strength of the paper is that many of the earlier findings (Figures 1-6) are strongly supported using independent lines of experimental evidence. However, I do have some major concerns regarding the interpretation of some of the later experimental

data and thus the justification of some key findings in the paper.

For example:

1. the finding that KLHL22 conjugates K48-linked ubiquitin chains to IRF7 leading to its proteasomal degradation and a reduction of IFN-I.

- Re: Supp 5A, the authors state in line 335 'in contrast to the other 3 proteins, KLHL22 expression obviously downregulated the protein level of IRF7, and this downregulation was reversed by MG132 treatment'.
I would disagree that any downregulation here is 'obvious' - it is perhaps less than 2-fold that of the MG132-treated sample and this margin is confounded by the slight but inconsistent levels of KLHL22 between the MG132-treated/untreated samples and also the loading control actin.
-Figures 7H and Supp 5D are used as evidence to suggest that IRF7 degradation relies on K48-linked ubiquitination by KLHL22. However, the evidence is not convincing. In Figure 7H, there is only an incremental difference in K48-linked ubiquitination of IRF7 +/- FLAG-KLHL22. In Supp 5D, there is no WT Ub control for comparison to the Ub mutants. Even without this essential control K48 ubiquitination does not appear to be blocked by using the K48R ubiquitin mutant.

2. The finding that 'KLHL22 ubiquitinates IRF7 directly and inhibits IFN-I induction' (Line 330)

Perhaps this statement needs to be revised to avoid the misinterpretation that there is a link between KLHL22 ubiquitination of IRF7 and inhibition of IFN-I. Figure 7J looks at the effect of nine IRF7 lysine mutants on KLHL22-mediated ubiquitination and six of these mutants were assessed for KLHL22-mediated IFN-I impairment (Figure 7K). While most of the mutants showed reduced ubiquitination, not all of these mutants impaired IFN-I induction. Also, why are K373R, K375R and K446R missing in Figure 7K? Currently, the implied link between KLHL22 ubiquitination of IRF7 and inhibition of IFN-I induction is not supported.

It would be good to compare IRF7 ubiquitination levels in WT MEFs vs KLHL22-KO MEFs.

Minor points
There are some grammatical issues.

Please define the BCR E3 ligase complex in the first instance.

KLHL22 is referred to throughout the text as if it is an E3 ligase - eg. line 330 'KLHL22 ubiquitinates IRF7 directly and inhibits IFN-I induction'. KLHL22 is an adaptor protein of the BCR E3 ligase complex, therefore KLHL22 is not an E3 ligase itself and cannot ubiquitinate. It should correctly be referred to as the BCR(KLHL22) ubiquitin ligase.

Line 115 - KLHL22 is incorrectly referred to as a substrate of BCR. It is rather the substrate recognition component.

The mass spectrum data (lines 145, 331) detailing putative IRF7-interacting proteins and ubiquitination-related proteins that interact with IRF7 has not been made available.

Line 137 ' a significant accumulation of IRF7 was observed after treatment with MG132'. The word 'significant' is a bit of a stretch here. There is an increase.

Line 218 - 'although IFN-b production continued to increase' - this statement implies that IFN-b was examined at various time points throughout the course of infection. Figure 3I shows the flu infection over a course of at least 6 days, but flu copies/ml in the lung (Figure 3J) and IFN-b (Figure 3K) was only monitored 2 days post-infection. To support the statement, later time points of infection should be examined.

The methods suggest that VSV infections were carried out at a MOI of 1, whereas the figure 2 legend suggests the VSV-GFP MOI is 0.1 (line 841). Please clarify. There is also no information provided about VSV-GFP.

Plaque assays are mentioned on line 187, but there is no figure/table reporting pfu/mL and also no method provided for the plaque assay.

Supp 2C - please label the x-axis

Line 219 - the inflammatory bowel disease section here comes from left field. That aside, the authors conclude from their results 'that XAF1 deficiency is not essential for proinflammatory cytokine induction', but have not actually tested the mice for the level of any proinflammatory cytokines.

The interaction between XIAP1 and IRF7 has been demonstrated by overexpression and coIP. The coIP lacks FLAG alone and HA alone controls overexpressed with the respective partner to prove that the binding is specific for the protein and not the tag (eg. co-express HA alone with FLAG-XIAP). An endogenous coIP demonstrating the interaction would also support this finding,

as has been provided for XAF1/IRF7.

Referee #3:

The paper by Liu et al., describes the IRF7-driven type I IFN is regulated by a an ISG called XAF1 which associated with and inhibited XIAP-driven ubiquitination which in turn was mediated by KLHL22 through s ubiquitin-dependent pathway. This pathway ultimately impact IFN production and susceptibility to viral infection in mouse models. The study provides a very comprehensive analysis of the different mechanistic underpinnings of this pathway. induces XAF and neg correlates with IRF7 levels. There are some issues to address, nevertheless.

- For the viral infections, effects are not clear and perhaps not much in magnitude. What are the units of Y axis on fig 1 J-K? What does a level of 1.5X induction mean? Same is true for other figures..
- Is it raw numbers plotted or fold induction>? What are the basal levels - which must be cited below
- Fig F: 1A-C needs quantitation to be convincing.
- ALL other figures should be checked for labels and degree of change (e,g, inhibition by XIAP in 8A not convincing. These misgivings should be overcome with adequate replicate experiments and statistical analysis.

Referee #1:

Major points:

1. In fig1, authors identified the ARG1, which was an essential protein in M2 macrophage differentiation. Was there any difference in proportion of induced M2 macrophage between WT and XAF1 MKO?

Response: We thank the reviewer for this critical point. To address the reviewer's question, we performed additional experiments and found that the expression of ARG1 in IL-4-induced M2 macrophage was comparable between WT and XAF1^{MKO} mice, and similar results in other maker genes of M2 macrophages, such as MRC1, YM1 were obtained **(Fig EV1H)**.

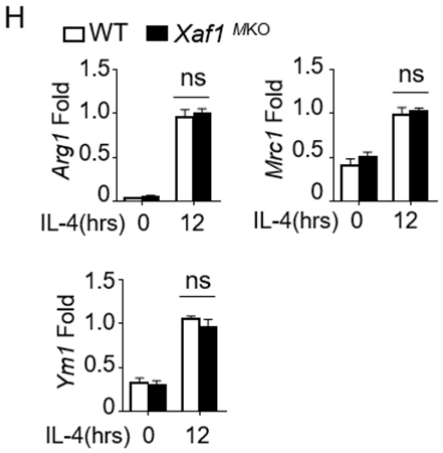

Figure EV1 (**H**) qRT-PCR analysis of ARG1, MRC1 and YM1 in IL-4 induced M2 macrophage in in WT and XAF1-deficient BMDMs. All data are representative of at least three independent experiments. Data in qPCR assay are presented as fold relative to the *Actin* mRNA level. Data are represented as the means ± SEMs. The significance of differences was determined by a t test.

7

2. When activated by viruses, IRF7 and IRF3 were phosphorylated and translocated to nuclear to promote transcription. It is known that phosphorylation of Serine 477/479 was essential for IRF7 activation (PMID:10893229), did the ubiquitination of IRF7 relied on its phosphorylation?

Response: This is a quite excellent question. Following reviewer's suggestion, we generated phosphorylation sites-mutated IRF7 S477/479A plasmid, and then we evaluated the ubiquitination level of WT IRF7 and IRF7 S477/479A. We found the IRF7 S477/479A mutant obviously displayed a decrease in the total and K48-linked ubiquitination level **(Fig EV4G-H)**. Furthermore, the inactive IRF7 S477/479A mutant was also destabilized in KLHL22-mediated ubiquitination as WT control, suggesting that ubiquitin-mediated degradation of IRF7 is not wholly dependent on direct IRF7 phosphorylation.

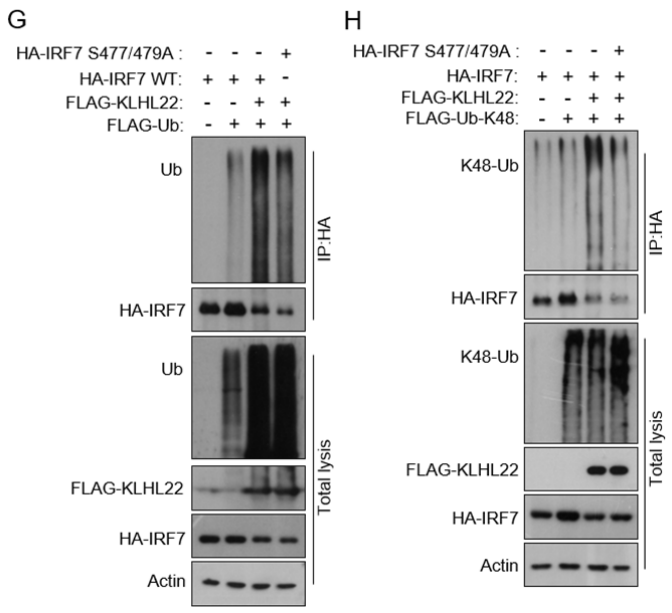

Figure EV4 (**G-H**) HEK293T cells were transfected with HA-tagged IRF7 WT and the inactive IRF7 S477/479A mutant. HA-tagged IRF7 was isolated by IP, and the ubiquitination level was then detected by IB. The data are representative of at least three independent experiments.

3. <u>When referred to K48 ubiquitination, authors should confirm the result using K48 point mutation ubiquitin. Of note, FigS5D did not represent the lysine point mutation but a screen for candidates. The author should supplement the data.</u>

Response: We thank the reviewer for this nice suggestion. We have performed the suggested experiment, and confirmed that KLHL22 specifically mediated K48-linked ubiquitination of IRF7 (**Fig 7H**).

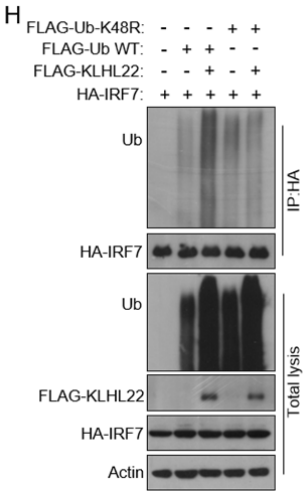

Figure 7 (**H**) HEK293T cells were transfected with IRF7 and FLAG-K48R-ubiquitin or FLAG-ubiquitin in the presence (+) or absence (–) of KLHL22 expression plasmids. HA-tagged IRF7 was isolated by IP, and the ubiquitination level was then detected by IB.

4. The authors should improve the ubiquitination of IRF7 mediated by KLHL22 in vitro.

Response: The reviewer's point is well taken. We performed the suggested experiment and purified recombinant IRF7 and BCR (BTB(KLHL22)-CUL3-RBX1) components. *In vitro* ubiquitination assay showed KLHL22-mediated IRF7 ubiquitination was obviously increased along with other BCR components (CUL3&RBX1), but not the six-repeats Kelch-deleted truncation **(Fig EV5A-B)**. This result was consistent with our data *in vivo*, and suggested that the six-repeats Kelch domain was necessary for KLHL22 to recognize IRF7 and mediate its proteasomal-dependent degradation.

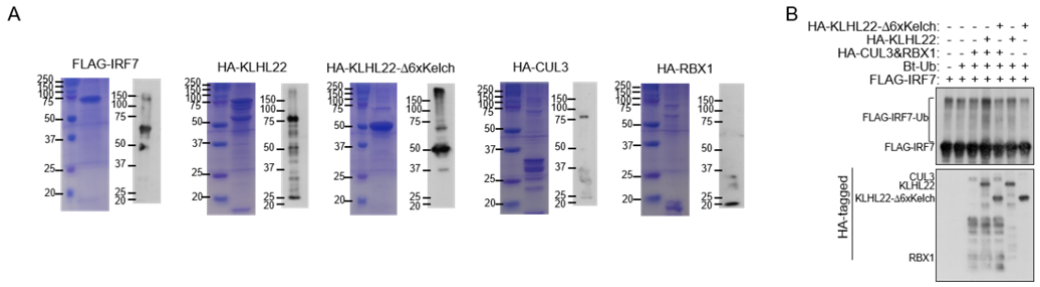

Figure EV5. KLHL22 interacts with IRF7 and promotes it ubiquitination. (**A**) purification of recombinant proteins was identified by Coomassie blue staining and IB. (**B**) CUL3-KLHL22 E3 ligase catalysed IRF7 ubiquitination in a cell-free system. Ubiquitin and recombinant FLAG-IRF7 were incubated with recombinant CUL3–RBX1 and KLHL22 or KLHL22-Δ6xKelch. The data are representative of at least three independent experiments.

Minor points:

The entire writing languages of this work should be improved, and color/scale bar of some figures were wrong.

Response: We thank the editors and reviewers for the constructive comments and suggestions. To improve the readability of our manuscript, we reorganize the data and correct typos with a language editing service.

1. ***In the page5, line 113. "We next confirmed that KLHL22 (a substrate of BCR) is an E3 ligase that directly targets IRF7" may should be changed to "We next confirmed that KLHL22 (an adaptor of BCR) is an E3 ligase that directly targets IRF7", which is more accurate and more consistent with the abstract section.***

Response: We apologize for ambiguous exposition, and we have made the correction and have revised it to "We next confirmed that KLHL22 (an adaptor of BCR) is an E3 ligase that directly targets IRF7".

2. ***In the page6, line 128. "we challenged wild-type (WT) bone marrow-derived macrophages (BMDMs) with Sendai virus (SeV)". The authors have already introduced the meaning of BMDM in the introduction section, so the abbreviation of BMDM can be used directly here.***

Response: We have carefully checked through our manuscript and have revised it to "we challenged wild-type (WT) BMDMs with Sendai virus…".

3. There are some typos and grammar errors in the text. For example, In the page6, line 130. "post stimulation" should be separated by spaces.

Response: We have checked through our manuscript and revised some typos and grammar errors.

4. In the page6, line 164. The abbreviation of "IFN-" should be unified.
In the page11, line 247. The abbreviation of "IRF7" should be unified.
In the page15, line 341. "KLHL22 is a substrate-specific adaptor of the BCR (BTB-CUL3-RBX1 (BCR) E3 ubiquitin ligase complex". The author expressed here repetition and cumbersomeness.

Response: We have checked through our manuscript and revised some format errors. We have carefully checked abbreviations included in our manuscript.

5. The labeling format of some figures is not uniform.
In the main Figure 7D. The minus sign should be changed to "-" instead of "_".
In the main Figure 7H. The abbreviations "K48-linked Ub" should be unified to "K48-Ub".

Response: We apologize for those format mistakes and we have revised and uniformed the labels.

6. It is best to mark the full name of each domain in the main Figure 6K.

Response: We have marked the full name of each domain (BIR: baculoviral IAP repeat domain；

UBA: ubiquitin-associated domain) in the main **Fig 6L**.

7. In the supplementary Figure 5C. The authors should label legend.

Response: We apologize for this format mistake and we have labeled legend.

8. Some protein bands, especially "Actin" bands, are overexposed and look too strong and a bit ugly. It is recommended to control the exposure time and adjust the sample load to obtain high-quality results.

Response: Following this excellent suggestion, we paid more attention to data quality by reducing sample loading or shortening exposure time.

9. The format of the histogram is not uniform, and the thickness of the lines is different. It is recommended to have a uniform format.

Response: We apologize for those format mistakes and we have checked through our manuscript and revised some format errors.

Referee #2:

The article by Liu et al uses a combination of in vivo experiments and in vitro studies and infections to report several findings centred around IRF7 protein stability including that 1) XAF1 interacts with IRF7, promoting K48-linked ubiquitination and proteasomal degradation of IRF7 to

restrict IFN-I induction 2) XIAP binds to IRF7 and inhibits IRF7 ubiquitination, promoting IFN-I induction and 3) KLHL22 conjugates K48-linked ubiquitin chains to IRF7 leading to its proteasomal degradation and a reduction of IFN-I. This work is potentially of great general interest as IRF7 is an interferon regulatory transcription factor of relevance to innate antiviral immunity and must be tightly regulated to avoid harming the host.

A strength of the paper is that many of the earlier findings (Figures 1-6) are strongly supported using independent lines of experimental evidence. However, I do have some major concerns regarding the interpretation of some of the later experimental data and thus the justification of some key findings in the paper.

1. the finding that KLHL22 conjugates K48-linked ubiquitin chains to IRF7 leading to its proteasomal degradation and a reduction of IFN-I.

- Re: Supp 5A, the authors state in line 335 'in contrast to the other 3 proteins, KLHL22 expression obviously downregulated the protein level of IRF7, and this downregulation was reversed by MG132 treatment'. I would disagree that any downregulation here is 'obvious' - it is perhaps less than 2-fold that of the MG132-treated sample and this margin is confounded by the slight but inconsistent levels of KLHL22 between the MG132-treated/untreated samples and also the loading control actin.

Response: We apologize for the low-quality data. Following this suggestion, we critically normalized the content of each plasmid for transfection and used a new batch of MG132. As shown in new **Fig EV4A**, we got a satisfied and obvious data that showed a clear and quantitated downregulation of IRF7. This data further confirmed our conclusion that "in contrast to the other 3 proteins, KLHL22 expression obviously repressed the protein level of IRF7, and this downregulation was reversed by MG132 treatment".

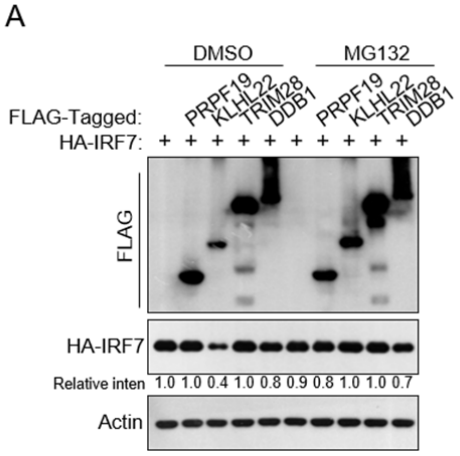

Figure EV4 (**A**) HEK293T cells were transfected with IRF7 and indicated expression plasmid. After treated with MG132, the indicated proteins were detected by IB. The data are representative of at least three independent experiments.

-Figures 7H and Supp 5D are used as evidence to suggest that IRF7 degradation relies on K48-linked ubiquitination by KLHL22. However, the evidence is not convincing. In Figure 7H,

<u>there is only an incremental difference in K48-linked ubiquitination of IRF7 +/- FLAG-KLHL22. In Supp 5D, there is no WT Ub control for comparison to the Ub mutants. Even without this essential control K48 ubiquitination does not appear to be blocked by using the K48R ubiquitin mutant.</u>

Response: We thank the reviewer for the critical thoughts and comments. To further support our conclusion, we have performed the additional experiments. We firstly found a decrease in total and K48-linked ubiquitination of IRF7 in *Klhl22* KO MEFs, compared to its WT control (**Fig 7G, Fig EV4D**).

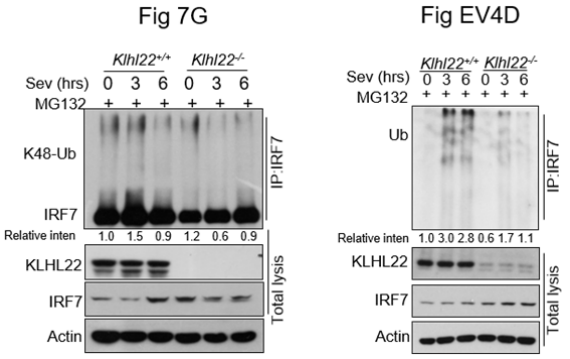

Figure 7 (**G**) After treatment with MG132, IRF7 was isolated by IP from WLs of KLHL22-KO MEFs and subjected to IB using anti-K48-ubiquitin. Total cell lysates were also subjected to direct IB.

Figure EV4 (**D**) After treatment with MG132, IRF7 was isolated by IP from WLs of KLHL22-KO MEFs and subjected to IB using anti-ubiquitin. Total cell lysates were also subjected to direct IB. The data are representative of at least three independent experiments.

Furthermore, we found KLHL22 (BCR)-mediated ubiquitination of IRF7 could be blocked by using mutated K48R ubiquitin **(Fig 7H and Figure only showed to reviewer)**.

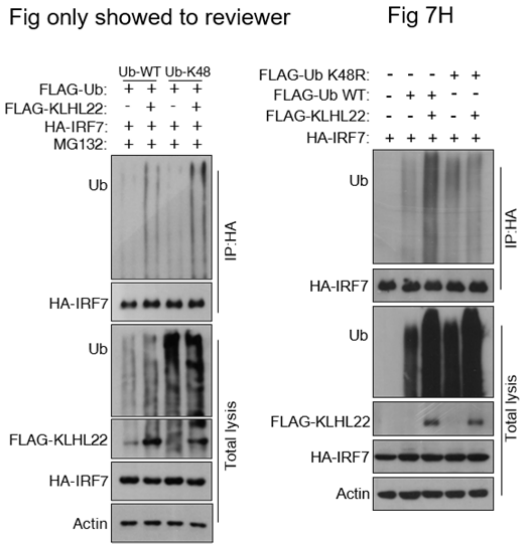

Figure 7 (**H**) HEK293T cells were transfected with IRF7 and FLAG-K48R-ubiquitin or FLAG-ubiquitin in the presence (+) or absence (–) of KLHL22 expression plasmids. HA-tagged

IRF7 was isolated by IP, and the ubiquitination level was then detected by IB. The data are representative of at least three independent experiments.

2. The finding that 'KLHL22 ubiquitinates IRF7 directly and inhibits IFN-I induction' (Line 330) Perhaps this statement needs to be revised to avoid the misinterpretation that there is a link between KLHL22 ubiquitination of IRF7 and inhibition of IFN-I. Figure 7J looks at the effect of nine IRF7 lysine mutants on KLHL22-mediated ubiquitination and six of these mutants were assessed for KLHL22-mediated IFN-I impairment (Figure 7K). While most of the mutants showed reduced ubiquitination, not all of these mutants impaired IFN-I induction. Also, why are K373R, K375R and K446R missing in Figure 7K? Currently, the implied link between KLHL22 ubiquitination of IRF7 and inhibition of IFN-I induction is not supported.

Response: We apologized for this misleading statement and we have revised it to "KLHL22 (BCR) ubiquitinates IRF7 directly and thus inhibits IFN-I induction". It's true there was a link between KLHL22 (BCR) ubiquitinates IRF7 and inhibits IFN-I induction. We observed a substantial increase in IFN-I production in KLHL22-KO MEFs upon polyI:C or SeV stimulation (**Fig 7E**). Moreover, an elevated IRF7 protein level was detected in KLHL22-deficient MEFs, suggesting a more stable state of IRF7 (**Fig 7F**). Notably, IRF7 degradation relied on K48-linked ubiquitination **(Fig 7G, Fig EV4D-E)**, and it could be blocked using mutated K48R ubiquitin **(Fig 7H, Fig EV4F)**. Therefore, we made conclusion that KLHL22 (BCR) directly targeted IRF7 to catalyze its K48-linked ubiquitination and proteasomal degradation, thus inhibited IFN-I induction.

As for identifying ubiquitination sites of IRF7 induced by KLHL22 (BCR), we generated a series of point mutants at the ID and DBD of IRF7 in which lysine (K) residues were replaced with arginine (R) residues. We repeated this experiment for several times and these six mutants in **Fig EV5E** displayed a stable and consistent result in each experiment. To address the reviewer's concern, we repeated this experiment by using K48-ubiquitin. Consistently, some mutants still showed weakly reduced ubiquitination, but only the K444R and K452R IRF7 mutants were more obviously reduced in KLHL22 (BCR)-mediated K48-linked ubiquitination (**Fig 7J**). We also included and tested the effects of other three mutants of IRF7 (K373R, K375R and K446R) for KLHL22 (BCR)-mediated IFN-I impairment (**Fig 7K**). The results still supported our previous conclusion that only the K444R and K452R IRF7 mutants were resistant to the effects of KLHL22 (BCR)-mediated IFN-I impairment.

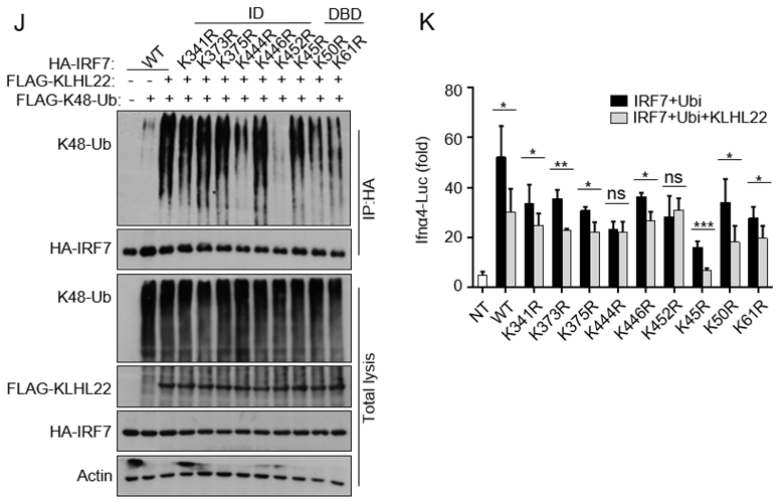

Figure 7 (**J**) HEK293T cells were transfected with KLHL22, FLAG-K48-ubiquitin and various IRF7 point mutants. After treatment with MG132, IB of K48-Ub was performed followed by IP with an anti-HA antibody. (**K**) HEK293T cells were transfected with IFN-α4 luciferase reporters and the indicated expression plasmids. Luciferase assays were performed to determine fold changes with respect to the empty vector group. The data are representative of at least three independent experiments.

It would be good to compare IRF7 ubiquitination levels in WT MEFs vs KLHL22-KO MEFs.

Response: We have performed the suggested experiment. As expected, the ubiquitination level of IRF7 was obviously weakened in KLHL22-KO MEFs (**Fig EV4D**).

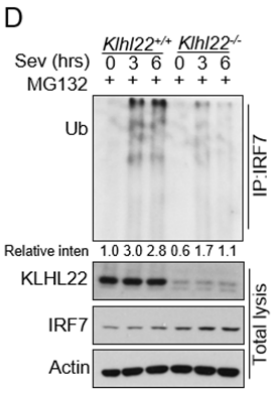

Figure EV4 (**D**) After treatment with MG132, IRF7 was isolated by IP from WLs of KLHL22-KO MEFs and subjected to IB using anti-ubiquitin. Total cell lysates were also subjected to direct IB. The data are representative of at least three independent experiments.

Minor points

There are some grammatical issues.

Response: We apologize for the poor English writing, and we have made the language polished by English editing companies.

Please define the BCR E3 ligase complex in the first instance.

Response: Following the reviewer's suggestion, we have defined the BCR E3 ligase complex in the introduction part in the first mention.

KLHL22 is referred to throughout the text as if it is an E3 ligase - eg. line 330 'KLHL22 ubiquitinates IRF7 directly and inhibits IFN-I induction'. KLHL22 is an adaptor protein of the BCR E3 ligase complex, therefore KLHL22 is not an E3 ligase itself and cannot ubiquitinate. It should correctly be referred to as the BCR(KLHL22) ubiquitin ligase.

Response: Reference to previous literature, we have made the correction and have revised it to "CUL3-KLHL22 E3 ligase".

Line 115 - KLHL22 is incorrectly referred to as a substrate of BCR. It is rather the substrate recognition component.

Response: We apologize for ambiguous exposition, and we have made the correction and have revised it to "We next confirmed that KLHL22 (an adaptor of BCR) is an E3 ligase that directly targets IRF7".

The mass spectrum data (lines 145, 331) detailing putative IRF7-interacting proteins and ubiquitination-related proteins that interact with IRF7 has not been made available.

Response: Following the reviewer's suggestion, we included supplementary Table 2 in the results and M&M section.

Line 137 ' a significant accumulation of IRF7 was observed after treatment with MG132'. The word 'significant' is a bit of a stretch here. There is an increase.

Response: We apologize for imprecisely statement and we revised it as "An increase in accumulation of IRF7…".

Line 218 - 'although IFN-b production continued to increase' - this statement implies that IFN-b was examined at various time points throughout the course of infection. Figure 3I shows the flu infection over a course of at least 6 days, but flu copies/ml in the lung (Figure 3J) and IFN-b (Figure 3K) was only monitored 2 days post-infection. To support the statement, later time points of infection should be examined.

Response: We apologize for inaccurate words. Our statement here means "Under XAF1 knockout background, deletion of IFNAR did not affect elevated expression of IFN-β". To clarify the statement, we replaced the "continued" with "still".

When referred to virus challenge, the *Ifnar*$^{-/-}$ mice exhibited shorter lifespan as shown in **Figure 3I**. On the other hand, during the severe symptom of mice on the late stage, adaptive immunity may be involved in. Therefore, we only monitored the flu copies and IFN-b induction on days 2 post virus challenge, which was consistent with various previous literatures.

The methods suggest that VSV infections were carried out at a MOI of 1, whereas the figure 2 legend suggests the VSV-GFP MOI is 0.1 (line 841). Please clarify. There is also no information provided about VSV-GFP.

Response: We apologize for misleading description. Actually, two types of VSV, including VSV-WT and VSV-GFP were used in our experiments. We used 0.1 MOI of VSV-GFP in Figure 2C, and used VSV-WT for other experiment with the dose of 1 MOI. We include this information in Figure legends and M&M section.

Plaque assays are mentioned on line 187, but there is no figure/table reporting pfu/mL and also no method provided for the plaque assay.

Response: Actually, the infected (GFP^+) cells were visualized under a fluorescence microscope (**Fig 2C, left**) and quantified by flow cytometry (**Fig 2C, right**). We apologized again for this mistake and we have revised our manuscript.

Supp 2C - please label the x-axis

Response: We are sorry for this missing label. We have labeled the x-axis (left: WT, right: $XAF1^{MKO}$).

Line 219 - the inflammatory bowel disease section here comes from left field. That aside, the authors conclude from their results 'that XAF1 deficiency is not essential for proinflammatory cytokine induction', but have not actually tested the mice for the level of any proinflammatory cytokines.

Response: We apologize for this negligence and we supplemented the data as reviewer's suggestion. As showed in **Fig EV2D**, there was no difference of these proinflammatory cytokines induction in DSS-induced WT and $XAF1^{MKO}$ mice.

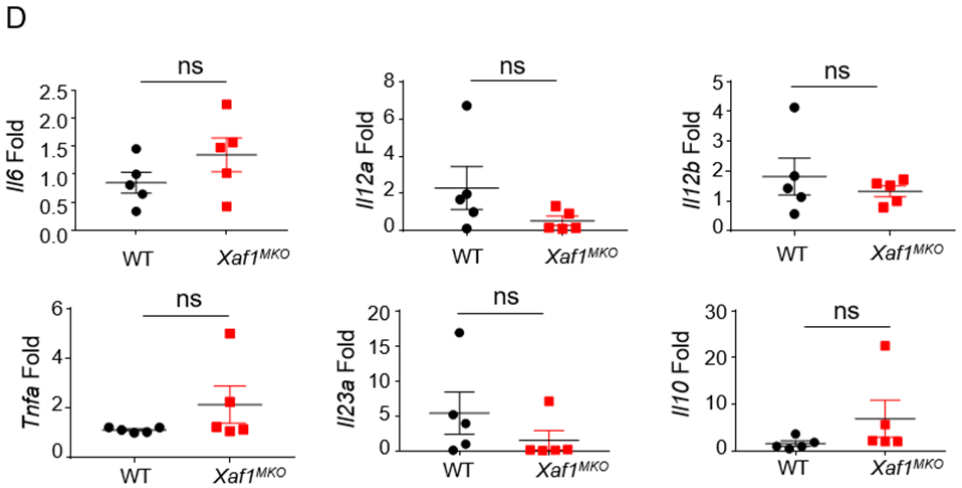

Figure EV2 In DSS-induced colitis model, proinflammatory cytokine production (**D**) of DSS-induced WT and $XAF1^{MKO}$ mice on day 8. Data are represented as the means ± SD. The significance of differences was determined by a t test.

<u>The interaction between XIAP1 and IRF7 has been demonstrated by overexpression and coIP. The coIP lacks FLAG alone and HA alone controls overexpressed with the respective partner to prove that the binding is specific for the protein and not the tag (eg. co-express HA alone with FLAG-XIAP). An endogenous coIP demonstrating the interaction would also support this finding, as has been provided for XAF1/IRF7.</u>

Response: The reviewer's point is well taken. Actually, we have the HA alone control in this result, but cut it off. We represented this data with the whole bands in **Fig 6L**.

For endogenous coIP, there is a technical issue that the molecular weight of endogenous IRF7 is 56KD that is quite similar as heavy chain of rabbit regulatory antibody. We thus overexpressed FALG-XIAP in MEFs, and recognized an obviously endogenous interaction between XIAP and IRF7 under SeV stimulation (**Fig 6K**).

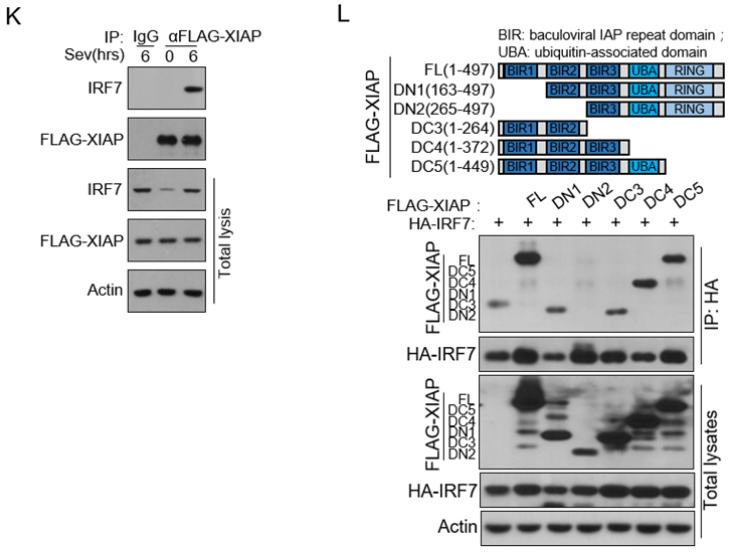

Figure 6 (**K**) The interaction between XIAP and IRF7 was assessed in XIAP overexpression MEFs activated by Sev. WLs were subjected to IP using an anti-FLAG-XIAP or anti-lgG antibody and then to IB and detected with the anti-IRF7 antibody. (**L**) The associations between IRF7 and various XIAP truncation mutants were detected through IP and IB. The data are representative of at least three independent experiments.

Referee #3:

The paper by Liu et al., describes the IRF7-driven type I IFN is regulated by a an ISG called XAF1 which associated with and inhibited XIAP-driven ubiquitination which in turn was mediated by KLHL22 through s ubiquitin-dependent pathway. This pathway ultimately impact IFN production and susceptibility to viral infection in mouse models. The study provides a very comprehensive analysis of the different mechanistic underpinnings of this pathway. induces XAF and neg correlates with IRF7 levels. There are some issues to address, nevertheless.

<u>- For the viral infections, effects are not clear and perhaps not much in magnitude. What are the units of Y axis on fig 1 J-K? What does a level of 1.5X induction mean? Same is true for</u>

Dear Prof. Li,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the two referees that I asked to re-evaluate your study, you will find below. As you will see, both referees now fully support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requested ask you to address in a final revised version of the manuscript:

- Please provide the abstract written in present tense throughout.

- Please have the final manuscript text carefully proofread by a native speaker. There are typos and a few grammatical errors present.

- 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 (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. There are still diagrams without statistics present (e.g. 3A,B,F) or diagrams with partial statistics.

- 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.

- The figures in the Appendix are rather small. Could you provide larger images?

- Please remove the paragraph 'Supporting online material' from the manuscript text file.

- It seems you used live virus strains in this study. Please add a paragraph to the methods section (titled 'Biosafety') providing details on where and how biosafety-relevant experiments were performed and that these were approved, and by whom (institution, government).

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

- You indicate in the author checklist that human participants or samples have been used. If this is the case, please supply information on the use of human material in the Materials & Methods section and provide proof of ethics approval as indicated in the checklist. If no human participants have been involved, please remove that part from the checklist (changing the entry to 'Not Applicable').

- You also indicate in the checklist that you used a select agent (a biological agent or a toxin has the potential to pose a severe threat) in the study (https://www.selectagents.gov/sat/list.htm). If this is true, please declare which agents have been used, that security measures have been applied and which ones, and that you have approval for the use (institutional, governmental). If select agent has been used, please remove that part from the checklist (changing the entry to 'Not Applicable').

In addition, I would need from you:
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- three to four short bullet points highlighting the key findings of your study (two lines each).
- 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:

I have no more concerns.

Referee #2:

The manuscript is much improved and the authors have adequately addressed each of my concerns.

The authors have addressed all minor editorial requests.

Prof. Yi-yuan Li
Southeast University
Key Laboratory for Developmental Genes and Human Disease, Ministry of Education, Institute of Life Sciences, Jiangsu Province High-Tech Key Laboratory for Bio-Medical Research, Southeast University
Nanjing, Jiangsu 210096
China

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The data shown in figures should satisfy the following conditions:

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- 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?
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  - definition of error bars as s.d. or s.e.m.

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Microbes: provide species and strain, unique accession number if available, and source.	Yes	Methods

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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	figure legend

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	figure legend
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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.	Yes	Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
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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)
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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	
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