EMBO Molecular Medicine

ZAKa/P38 kinase signaling pathway regulates hematopoiesis by activating the NLRP1 inflammasome

Lola Rodriguez-Ruiz, Juan Lozano-Gil, Elena Naranjo-Sánchez, Elena Martínez-Balsalobre, Alicia Martinez-Lopez, Christophe Lachaud, Miguel Blanquer, Toan Phung, Diana García-Moreno, Maria L. Cayuela, Sylwia Tyrkalska, Ana Perez-Oliva, and Victoriano Mulero
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(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.)

12th Jul 2023

Dear Prof. Mulero,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the two reviewers who agreed to evaluate your manuscript. Both referees recognize potential interest of the study but also raise important criticism that should be addressed in a major revision. If you would like to discuss further the points raised by the referees, I am available to do so via email or video. Let me know if you are interested in this option.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

*****

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

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

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/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/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and

database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). See also 'Figure Legend' guidelines: https://www.embopress.org/page/journal/17574684/authorguide#figureformat

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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

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

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

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

See detailed instructions here:

.

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- the results obtained and

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12) For more information: There is space at the end of each article to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

13) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

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***** Reviewer's comments *****

Referee #1 (Remarks for Author):

Rodriquez-Ruiz and colleagues present data regarding NLRP1 activation in zebrafish, related to LRRFIP1/FLII and ZAKa, with relevance for the system in humans. Overall there are a lot of separate parts to this investigation, and in many places additional controls could be warranted. To some extent, the LRRFIP1/FLII and ZAKa parts seem separable, but with some additional in vitro analysis may link together more clearly.

Major points

The epistasis experiment showing enforced FLII does not reduce neutrophil numbers from LRRFIP1 deficient fish is very interesting. Previously FLII was reported to inhibit Caspase-1 (PMID: 18411310), can the authors determine why that does not occur in this context? Is BCAP involved or dispensable for the effect of FLII on NLRP1 in zebrafish?

It seems somewhat surprising that agents triggering ribotoxic stress such as ansiomycin had effects consistent with NLRP1 activation in vivo when used systemically. In many cell types, this type of insult results in apoptosis, as opposed to pyroptosis, and it may have been expected to be toxic when used in vivo at concentrations that activate NLRP1.

The experiments with overexpressed NLRP1 (S107D/A) appear straightforward, however the interpretation seems challenging and somewhat indirect. As the conclusion appears to be that LRRFIP1/FLII inhibit NLRP1 by preventing phosphorylation of S107, perhaps it would be best to try and document this formally with biochemical analysis in vitro?

Referee #2 (Comments on Novelty/Model System for Author):

See my review.
Technical quality - overall it is high, but although there is biological replication within data presented (for example, there are multiple embryos), there is paucity of information about experimental replication (whether the comparison or intervention was performed more than once). This is one of major points to the authors.
Novelty - to my knowledge, the pathway assembled is new
Medical impact - there is potential medical impact. The implication for ribosomal-stress diseases is based on a single cell line + chemical treatment model. The implication for inflammatory diseases is based on an in vivo model. A repurposed drug is evaluated.
Adequacy of the model system - the use of a human cell line and zebrafish models is a strength of the paper. It has provided opportunity for biochemical studies particularly in the human cell line system, and correlative genetic studies in both systems.
Re (5) clarity and Interest for the nonspecialist - the genes involved have impossible non-intuitive names and abbreviations that provide little help for the non-specialist to understand the pathway being assembled; I have suggested a pathway diagram among the main figures (most likely it will be the last figure).

Referee #2 (Remarks for Author):

The manuscript of Rodriguez-Ruiz et al presents a comprehensive set of experiments that implicate the NLRP1 inflammasome in influencing the erythroid/myeloid balance of haematopoietic output from HSCs. Molecular mechanistic studies link LRRFIP1 and FLII, and the ZAKalpha/P38 kinase pathway.

Overall the manuscript is comprehensive, logical and well-presented. The scientific logic and the evidence for the genetic and molecular mechanisms are thorough, of high technical quality, and supported by the evidence presented.

Major comments

1. The gene editing efficacy analysis shows that the crRNAs designed for either lrrfip1a and lrrfip1b editing each actually directed editing of both lrrfip1a and lrrfip1b at approx. equal efficiencies, but overall to a different degree (20% and 20%, and 65% and 85% respectively). While the text correctly acknowledges this by referring to the larvae as deficient in both paralogs (as in lines 168-169), the x-axis labelling within the figures (e.g. as in Figure 4) uses crRNA design intent rather than the actual edited outcome. This is somewhat misleading, and will be so especially to readers who may survey the figures and not read the very fine detail of the narrative text. The author's already provide a solution for this in Fig 4F. Following that precedent, these two crRNAs would be better labelled "crRNA irrfip1a/b-1" and "crRNA irrfip1a/b-2" to reflect what they did, rather than what they were designed to do, throughout the entire paper.

2. Throughout the paper, it not clear what degree of replication was behind the experiments. While the plots indicate the number of zebrafish embryos evaluated, it is never clear how many times an experiment was independently performed from start to finish. For example, in Fig 4 (all panels) were the groups in one panel from a single parallel study? Were any panels compiled as composites from non-parallel experiments? Did all the embryos represented in one outcome result from a single run or from multiple runs of the experiment? In panel H, how many independent times was the drug treatment applied and this outcome observed? For such an experiment, the usual expectation would be n=3 independent replicates, independent of the total number of individual embryos in each iteration of the assay. Similarly, the replication of all biochemical assays such as Western blots, IPs etc is not clear. In Fig 3, the variation appears to be from technical replicates within one run of an in vitro experiment.

3. The testing of interventions based on these new mechanistic insights to intentionally alter hematopoietic outcomes in K562 cells and the zebrafish spint1a mutant model is a particularly interesting aspect of this report. This certainly adds to the significance of the report, and in the discussion the consideration if this is appropriately qualified. While these data support the claim of the final abstract sentence (lines 44-47) that they "reveal" "novel therapeutic strategies", this seemed too strong. That the insights from this work might help rare ribosomal-stress diseases (such as Diamond-Blackfan anemia) is hypothesised, suggested, or a possibility for the future - suggest the use of more qualified language.

4. I suggest that many readers' understanding of the mechanism in paper would be greatly assisted by having the overall molecular mechanism summarised in a figure within the main paper, rather than just in supplementary material.

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2. Line 111. From the methods sections and all the data presented, it appears that mfap4-driven reporter lines were used for studies of macrophage lineage cells, not mpeg1-driven reporter lines as stated here.

3. Figure S1B - there are 46 genes listed, not 50 as stated in the legend.

4. Almost all figures and supplementary figures with photomicrographs. Scale bars are labelled "uM" (which means micro-moles/liter) instead of "um" for micrometres (for example, it is incorrect in Fig 1D; it is correct in Figure 3B-C). In figures such as Fig 1D, it is confusing, because there is also the concentration of Hemin to consider, which was 50 microM.

5. Nomenclature. It is acknowledged that in a paper that uses a human cell line and zebrafish in vivo models it is very complex to apply an absolutely precise gene/RNA/protein capitalisation/upper-lower case punctuation nomenclature convention - some generic text applying to both species cannot be avoided. However, there is still a need for greater consistency in some places. Lines 158-175 describing the generation of the gene edited lines correctly introduce the zebrafish gene names as lower-case italicised (line 161). The multiple mentions of the target in lines 163-165 are target genes, and should be italics. The resultant lines as "Lrrfip1a-deficient" etc, a nomenclature convention indicating that they are deficient in the protein, although this has not been experimentally demonstrated (there is precise attention to what occurred at the genomic (DNA) and transcriptomic (RNA) levels, but the not protein level). Make this consistent here, and check these nomenclature conventions throughout the manuscript.

6. There are also inconsistencies though the figure labels whether the reporter line is (Lyz:dsRED) or (lyz:dsRED), (Mfap4:tomato) or (mfap4:tomato) - lower case is correct.

7. Tables S1 and S2 - despite specifically referring to use of recommended nomenclature guidelines, several gene names are inconsistently formatted between the two tables.

8. Fig7K, Fig 8H y-axis - adding "CASP1" to the label would be helpful (as in Fig 1I).

9. Figure S1A - hybridisation (NOT hibridation). Whether "labelling" and "labelled" should have -l- or -ll- is confusing, but use either one or the other consistently, not both.

10. Fig S12G. Graphics incomplete.

11. Line 855. Should be Figure S2 (NOT Figure 2).

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13. Line 265 "fantastic" - this is an informal use of "fantastic" to mean "very very good" - "uniquely appropriate" or similar would be better.

Referee #1 (Remarks for Author):

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The epistasis experiment showing enforced FLII does not reduce neutrophil numbers from LRRFIP1 deficient fish is very interesting. Previously FLII was reported to inhibit Caspase-1 (PMID: 18411310), can the authors determine why that does not occur in this context? Is BCAP involved or dispensable for the effect of FLII on NLRP1 in zebrafish?
This is an interesting point. As we discussed in the manuscript, LRRFIP2 directly interact and inhibit the activation of macrophage NLRP3 inflammasome by recruiting FLII and facilitating the inhibition of caspase-1. However, LRRFIP1 is unable to interact with NLRP3. However, our biochemical and functional results show that a similar mechanism operates to inhibit the NLRP1 inflammasome but mediated by LRRFIP1.

Although it would be interesting to address whether BCAP is required for the negative regulation of NLRP1 inflammasome by LRRFIP1/FLII in future studies, we did not find BCAP in our MS analysis. However, we find novel proteins and recently reported to regulate NLRP1 inflammasome activity, such as thioredoxin (PMID: 36332009). However, we have discussed these points in the revised manuscript (lines 322-324 and 329-333).

It seems somewhat surprising that agents triggering ribotoxic stress such as ansiomycin had effects consistent with NLRP1 activation in vivo when used systemically. In many cell types, this type of insult results in apoptosis, as opposed to pyroptosis, and it may have been expected to be toxic when used in vivo at concentrations that activate NLRP1.
We agree on this point. The concentrations used of anisomycin activates Nlrp1 inflammasome in zebrafish larvae and alters hematopoiesis. However, we did not find any toxicity or effect on development. This suggests that erythropoiesis is highly affected by translation efficiency of ribosomes, and this is consistent with ribosomopathies, such as DBA, where *GATA1* translation is impaired.

The experiments with overexpressed NLRP1 (S107D/A) appear straightforward, however the interpretation seems challenging and somewhat indirect. As the conclusion appears to be that LRRFIP1/FLII inhibit NLRP1 by preventing phosphorylation of S107, perhaps it would be best to try and document this formally with biochemical analysis in vitro?
Our functional studies in zebrafish demonstrate that the phosphorylation of human NLRP1 at S107 by the Zaka/P38 kinase axis is essential to regulate hematopoiesis and that the mechanism is conserved between zebrafish and human. We did not conclude

that LRRFIP1/FLII regulate NLRP1 activation by preventing phosphorylation of S107.It is presumably that LRRFIP1/FLII inhibit caspase-1 downstream NLRP1 inflammasome activation, since FLII is a caspase-1 pseudosubstrate, although other mechanisms may also operate since LRRFIP1 and FLII block NLRP1-induced ASC speck formation in HEK293 cells. Anyway, demonstrating whether LRRFIP1/FLII inhibit the phosphorylation of NLRP1 is technically challenging, as it would require the production of recombinant NLRP1, P38, LRRFIP1 and FLII for performing radiometric in vitro kinase assays (PMID: 36315050). Therefore, we think this study requires a great effort and the proposed mechanism is not supported by experimental evidence. However, we have discussed this point in the revised manuscript (lines 333-337).

Referee #2 (Remarks for Author):

The manuscript of Rodriguez-Ruiz et al presents a comprehensive set of experiments that implicate the NLRP1 inflammasome in influencing the erythroid/myeloid balance of haematopoietic output from HSCs. Molecular mechanistic studies link LRRFIP1 and FLII, and the ZAKalpha/P38 kinase pathway.

Overall the manuscript is comprehensive, logical and well-presented. The scientific logic and the evidence for the genetic and molecular mechanisms are thorough, of high technical quality, and supported by the evidence presented.
We are pleased with the reviewer's comments on our manuscript.

Major comments

1. The gene editing efficacy analysis shows that the crRNAs designed for either lrrfip1a and lrrfip1b editing each actually directed editing of both lrrfip1a and lrrfip1b at approx. equal efficiencies, but overall to a different degree (20% and 20%, and 65% and 85% respectively). While the text correctly acknowledges this by referring to the larvae as deficient in both paralogs (as in lines 168-169), the x-axis labelling within the figures (e.g. as in Figure 4) uses crRNA design intent rather than the actual edited outcome. This is somewhat misleading, and will be so especially to readers who may survey the figures and not read the very fine detail of the narrative text. The author's already provide a solution for this in Fig 4F. Following that precedent, these two crRNAs would be better labelled "crRNA irrfip1a/b-1" and "crRNA irrfip1a/b-2" to reflect what they did, rather than what they were designed to do, throughout the entire paper.
Thanks for this observation. In Figs 4F and S14I, we used both gRNAs together. Therefore, to clarify this issue we have include a note in the legends of the affected figures which reads: "Note that either *lrrfip1a* or *lrrfip1b* crRNAs/Cas9 complexes target both *lrrfip1* paralogs (see Appendix Figure S10).".

2. Throughout the paper, it not clear what degree of replication was behind the experiments. While the plots indicate the number of zebrafish embryos evaluated, it is never clear how many times an experiment was independently performed from start to finish. For example, in Fig 4 (all panels) were the groups in one panel from a single parallel study? Were any panels compiled as composites from non-parallel experiments? Did all the embryos represented in one outcome result from a single run or from multiple runs of the experiment? In panel H, how many independent times was the drug treatment applied and this outcome observed? For such an experiment, the usual expectation would be n=3 independent replicates, independent of the total number of individual embryos in each iteration of the assay. Similarly, the replication of all biochemical assays such as Western blots, IPs etc is not clear. In Fig 3, the variation appears to be from technical replicates within one run of an in vitro experiment.
We have clarified this in the M&M section which now reads: " At least 3 independent experiments were performed with zebrafish larvae and biochemical studies. Three independent caspase-1 activity assays were performed in all experiments using a pool of 30 larvae and one representative experiment is shown with 3 technical replicates. All larvae from the different independent experiments were pooled for plotting and statistical analysis. The number of total larvae analyzed in each experiment is indicated in all figures". Therefore, Fig 4H shows a pool of larvae from 3 independent

experiments and Fig 3 shows the quantification of specks from 3 independent experiments.

3. The testing of interventions based on these new mechanistic insights to intentionally alter hematopoietic outcomes in K562 cells and the zebrafish spint1a mutant model is a particularly interesting aspect of this report. This certainly adds to the significance of the report, and in the discussion the consideration if this is appropriately qualified. While these data support the claim of the final abstract sentence (lines 44-47) that they "reveal" "novel therapeutic strategies", this seemed too strong. That the insights from this work might help rare ribosomal-stress diseases (such as Diamond-Blackfan anemia) is hypothesised, suggested, or a possibility for the future - suggest the use of more qualified language.
We have rewritten this sentence to dampen our conclusion. It now reads: "In conclusion, our results reveal that the NLRP1 inflammasome regulates hematopoiesis and pave the way to develop novel therapeutic strategies for the treatment of hematopoietic alterations associated with chronic inflammatory and rare diseases."

4. I suggest that many readers' understanding of the mechanism in paper would be greatly assisted by having the overall molecular mechanism summarised in a figure within the main paper, rather than just in supplementary material.
Thanks for this suggestion. We have moved Fig S19 to the main figures and it is now Fig. 9.

Minor comments

1. Abstract (line 44) conclusion (NOT conclusions)
Corrected.

2. Line 111. From the methods sections and all the data presented, it appears that mfap4-driven reporter lines were used for studies of macrophage lineage cells, not mpeg1-driven reporter lines as stated here.
You are right; we used mfap4 line in this paper. It has been corrected.

3. Figure S1B - there are 46 genes listed, not 50 as stated in the legend.
Corrected.

4. Almost all figures and supplementary figures with photomicrographs. Scale bars are labelled "uM" (which means micro-moles/liter) instead of "um" for micrometres (for example, it is incorrect in Fig 1D; it is correct in Figure 3B-C). In figures such as Fig 1D, it is confusing, because there is also the concentration of Hemin to consider, which was 50 microM.
We have corrected them.

5. Nomenclature. It is acknowledged that in a paper that uses a human cell line and zebrafish in vivo models it is very complex to apply an absolutely precise gene/RNA/protein capitalisation/upper-lower case punctuation nomenclature convention - some generic text applying to both species cannot be avoided. However, there is still a need for greater consistency in some places. Lines 158-175 describing the generation of the gene edited lines correctly introduce the zebrafish gene names as lower-case italicised (line 161). The multiple mentions of the target in lines 163-165 are target genes, and should be italics. The resultant lines as "Lrrfip1a-deficient" etc, a nomenclature convention indicating that they are deficient in the protein, although this has not been experimentally demonstrated (there is precise attention to what occurred at the genomic (DNA) and transcriptomic (RNA) levels, but the not protein level). Make this consistent here, and check these nomenclature conventions

throughout the manuscript.
We have double checked and corrected them. We have also replaced deficient by crispant and use gene moneclature, for example "*nlrp1* crispant larvae".

6. There are also inconsistencies though the figure labels whether the reporter line is (Lyz:dsRED) or (lyz:dsRED), (Mfap4:tomato) or (mfap4:tomato) - lower case is correct.
We have corrected them.

7. Tables S1 and S2 - despite specifically referring to use of recommended nomenclature guidelines, several gene names are inconsistently formatted between the two tables.
In Table S1, primers for both human and zebrafish are shown. We have now clarified this.

8. Fig7K, Fig 8H y-axis - adding "CASP1" to the label would be helpful (as in Fig 1I).
Corrected.

9. Figure S1A - hybridisation (NOT hibridation). Whether "labelling" and "labelled" should have -l- or -ll- is confusing, but use either one or the other consistently, not both.
Corrected.

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Corrected.

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Corrected.

12. Fig 3B. Bold Hemin and Anysomicine labels for consistency.
Changed.

13. Line 265 "fantastic" - this is an informal use of "fantastic" to mean "very very good" - "uniquely appropriate" or similar would be better.

Replaced as suggested.

15th Aug 2023

Dear Prof. Mulero,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please address all the minor points raised by the referees.
2) In the main manuscript file, please do the following:
- Correct/answer the track changes suggested by our data editors by working from the attached document.
- Limit keywords to max. 5.
- Remove track changes.
- Please add callouts for Fig.6B and Fig. 9A-B.
- Please rename "Disclosure Statement" to "Disclosure Statement & Competing Interests". 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.
- Correct the reference citation in the reference list. Where there are more than 10 authors on a paper, 10 will be listed, followed by "et al.". Please check "Author Guidelines" for more information.
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3) Funding: Please merge it with "Acknowledgments" and make sure that information about all sources of funding are complete in both our submission system and in the manuscript. Currently MCIN/AEI/10.13039/501100011033, Juan de la Cierva-Incorporación postdoctoral contract, PhD fellowship to LR-R, ISCIII (Miguel Servet CP20/00028 and CP21/00028, Consejería de Salud de la CARM are missing in our submission system.
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8) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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2) Separate figure files*

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https://www.embopress.org/page/journal/17574684/authorguide#expandedview

4) a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word file).

5) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting
- the medical issue you are addressing,
- the results obtained and
- their clinical impact.
This may be edited to ensure that readers understand the significance and context of the research.
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7) Author contributions: the contribution of every author must be detailed in a separate section.

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***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System for Author):

Rodriquez-Ruiz and colleagues present data regarding NLRP1 activation in zebrafish, related to LRRFIP1/FLII and ZAKa, with relevance for the system in humans. Overall there are a lot of separate parts to this investigation, and I remain confused about the LRRFIP1/FLII data.

Referee #1 (Remarks for Author):

Two of my major points from the previous revisions remain only partially addressed.

1. Why enforced FLII did not reduce neutrophil numbers from LRRFIP1 deficient fish, there was no response.

3. Given that LRRFIP/FLII might inhibit either NLRP1/ASC spec formation, or Caspase-1 activity, some experiment that could distinguish between them in this context seems important. At least NLRP1 phosphorylation could be assessed in cell lysates using phos-tag gels or MS, which would not require radiometric in vitro kinase assays and helps link these parts of the manuscript together?

Referee #2 (Comments on Novelty/Model System for Author):

Technical quality has been improved in the review process.

Referee #2 (Remarks for Author):

The authors have addressed my previous major and minor comments satisfactorily, and I have noted the responses to the other reviewer's comments.

Congratulations to the authors for their interesting and well-presented work.

Remaining errors:
The authors clarified that only the Tg(mfap4:mCherry)line was used; the ump6 allele is listed in line 375. For this reason:
Figure 7, Panel I. Labelled Tg(mpeg:mCherry). I believe this should be Tg(mfap4:mCherry).
Figure S13, Panel C. Labelled Tg(mfap4:tomato). I believe this should be Tg(mfap4:mCherry).
Figure S14, Panel E. Tg(mfap4:mcherry). For consistency should be Tg(mfap4:mCherry) i.e. uppercase "C".

EDITOR

1) Please address all the minor points raised by the referees.

Please, see below.

2) In the main manuscript file, please do the following:
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All changes done. The callout for Figure 9 is in the Discussion section.

3) Funding: Please merge it with "Acknowledgments" and make sure that information about all sources of funding are complete in both our submission system and in the manuscript. Currently MCIN/AEI/10.13039/501100011033, Juan de la Cierva-Incorporación postdoctoral contract, PhD fellowship to LR-R, ISCIII (Miguel Servet CP20/00028 and CP21/00028, Consejería de Salud de la CARM are missing in our submission system.

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5) For more information: This space should be used to list relevant web links for further consultation by our readers. Could you identify some relevant ones and provide such information as well? Some examples are patient associations, relevant databases, OMIM/proteins/genes links, author's websites, etc...

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1. OMIM for Diamond-Blackfan anemia: https://www.omim.org/entry/105650
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Agree.

Referee #1 (Remarks for Author):

Two of my major points from the previous revisions remain only partially addressed.

1. Why enforced FLII did not reduce neutrophil numbers from LRRFIP1 deficient fish, there was no response.

As we have indicated in our manuscript, these epistasis studies suggest that the ability of Flii to inhibit the Nlrp1 inflammasome is dependent on Lrrfip1 (lines 187-188). WE have now also discussed this point in the revised version (lines 321-324).

3. Given that LRRFIP/FLII might inhibit either NLRP1/ASC spec formation, or Caspase-1 activity, some experiment that could distinguish between them in this context seems important. At least NLRP1 phosphorylation could be assessed in cell lysates using phos-tag gels or MS, which would not require radiometric in vitro kinase assays and helps link these parts of the manuscript together?

We have performed this experiment with phos-tag and, unfortunately, were unable to detect NLRP1 phosphorylation.

Referee #2 (Comments on Novelty/Model System for Author):

Technical quality has been improved in the review process.

Referee #2 (Remarks for Author):

The authors have addressed my previous major and minor comments satisfactorily, and I have noted the responses to the other reviewer's comments.

Congratulations to the authors for their interesting and well-presented work.

We thanks that our responses satisfy this reviewer.

Remaining errors:

The authors clarified that only the Tg(mfap4:mCherry)line was used; the ump6 allele is listed in line 375. For this reason:

Figure 7, Panel I. Labelled Tg(mpeg:mCherry). I believe this should be Tg(mfap4:mCherry).

Figure S13, Panel C. Labelled Tg(mfap4:tomato). I believe this should be Tg(mfap4:mCherry).

Figure S14, Panel E. Tg(mfap4:mcherry). For consistency should be Tg(mfap4:mCherry) i.e. uppercase "C".

We are sorry for this mistake. We have used the line Tg(mfap4.1:Tomato)^{xt12}. We have corrected all figures and the manuscript.

22nd Aug 2023

Dear Prof. Mulero,

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Of note, deposited microscopy images at BioStudies repository under the accession number S-BIAD823 are currently not accessible. Please be aware that all deposited data should be freely available upon publication.

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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Each figure caption should contain the following information, for each panel where they are relevant:
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- 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:
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Please detail **housing and husbandry conditions**.	Yes	Materials & Methods

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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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