EMBO reports

Lipin-2 Regulates the Antiviral and Anti-Inflammatory Responses to Interferon

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Thank you for the submission of your research manuscript to EMBO reports. I have already forwarded to you the reports from the three referees that were asked to evaluate your study (which can be found again at the end of this email).

The referees think that the study is of interest, but have several concerns, indicating that the study needs a major revision to be considered for publication. After cross-commenting with the referees and going through your preliminary point-by-point response (revision plan), I decided to proceed with the manuscript

Given the constructive referee comments, I 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 final detailed point-by-point response (as indicated in your revision plan). 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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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an

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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 now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. 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.

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. 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. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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Finally, please order the manuscript sections like this, using these names:

Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

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

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have been investigating the role of lipin-2 - encoded by Lpin2 (LPIN2) - in inflammatory and immune responses in bone marrow derived macrophages (BMDMs) and in human monocyte-derived macrophages. Lipin-2 is a phosphatidic acid phosphatase, which plays a central role in lipid metabolism. Its regulatory role in inflammation became apparent in a disease known as Majeed syndrome, which results from LPIN2 mutation and is characterized by recurrent flares of fever and inflammation in their joints and skin.

The authors previously demonstrated that lipin-2 inhibits the activation and sensitization of the purinergic receptor P2X7 and thus regulates NLRP3 inflammasome activation (PMID: 28031477).
In the present work, the authors provide substantial evidence that lipin-2 mRNA and protein expression is upregulated by interferons in a JAK1/2/STAT-1-dependent manner. Using a knockout-validated antibody, they show that lipin-2 protein is upregulated in response to toll-like receptor (TLR) agonists. The response is obviously mediated by interferons since IFN-ß and IFN-γ also activated lipin-2 mRNA expression. Moreover, TLR/IFN-ß-induced lipin-2 expression was not effective in cells deficient in the IFN-ß receptor subunit 1 (Ifnar1). The selective JAK1/2 inhibitor ruxolitinib completely abolished Lpin2 mRNA induction, as well as lipin-2 protein upregulation by IFN-ß. The role of STAT1 was established in gene array based experiments by demonstrating that IFN-γ induced lipin-2 mRNA did not occur in STAT1-deficient BMDMs. Analyses of ChIP-seq data from BMDMs stimulated with IFN-ß and IFN-γ suggested that STAT1 can bind to three different sequences in the locus of Lpin2.

The authors subsequently addressed the role of lipin-2 in the replication control of murine CMV, which is known to be IFN-dependent. For practical reasons, a murine CMV carrying the GFP gene was used for the infection of wildtype and lipin-2 deficient BMDMs. Macrophage lipin-2 deficiency caused a significant increase in fluorescence forming units in vitro. In addition, mice deficient lipin-2 had a higher viral load in liver and spleen. Importantly, lipin-2 had no effect in the control of vaccinia virus, which is IFN-independent.

Using the TLR3 ligand poly I:C as a viral nucleic acid mimetic and the P2X7 agonist ATP, the authors examined the role of lipin-2 in inflammasome activation including the release of mature bioactive IL-1ß. As expected, lipin-2-deficient BMDMs produced higher levels of IL-1ß. LPIN2 knockdown using RNA interference in primary human macrophages also increased IL-1ß, although the effect was modest. MAPK activation was accelerated in lipin-2-deficient macrophages and, consistent with current knowledge, IL-1ß production was found to mainly depend on MAPK p38 (PMID: 19740675). Moreover, the absence of lipin-2 results in an increase in NLRP3 and CASP1 (expression and activity) as well as of the TLR3 itself and certain immunostimulatory genes (ISGs). Enhanced inflammasome activation resulted in an increase in pyroptosis, an inflammatory form of cell death that depends on inflammasome activation. The authors examine the role of mtDNA release to the cytosol and the production of ROS during inflammasome activation and overproduction of IL-1ß in the absence of lipin-2.

Last but not least, the authors used publicly available databases of gene expression arrays to evaluate whether LPIN2 levels were altered in COVID-19 patients. They made the intriguing obeservation that patients with low LPIN2 had severe symptoms and were not able to recover from COVID19.

Comments:

This is a well-written, detailed and important study based on modern and sophisticated methods.

The role of STAT1 was established in gene array experiments. No inhibitors were tested. Is this due to the limited availability of inhibitors of this pathway? The authors may comment on this.

Why was IFN-γ used in the gene array experiments? IFN-ß? TLR agonists?

The text referring to Figure 5 requires correction. The authors do not refer to Fig. 5A, which shows BMDM data, at all in the text! The authors then state: "the same behavior was also found in primary human macrophages silenced for LPIN2 (Fig. 5B and C)." However, according to the legend to Figure 5, BMDM data are shown in Fig. 5B, while data from primary human macrophages are shown in Fig. 5C.

LPIN2 silencing in primary human macrophages (Fig. 5C) is not impressive. However, this is not surprising. RNA interference is difficult (if not impossible) in primary human macrophages. This may be due to the innate features of macrophages, which may respond to siRNA sequence-dependent delivery vehicles and even the RNAi process itself (PMID: 28386261) or to the "digestive" capacity of this cell type. In many human macrophage studies, RNA interference has therefore been performed in the THP-1 cell line.
If human primary macrophages would play a more significant role in this work, which is currently not the case, a minimal phenotype would be desirable (CD14, CD163, CD206), also because there are so many different protocols for macrophage generation (adherence, magnetic isolation, {plus minus} M-CSF, human vs. bovine serum, duration of culture).

What was the purpose of poly I:C transfection? A bit more explanation would be helpful.

Overall, this work is an important contribution, which may however benefit from some streamlining measures. The authors might consider whether all data (MAPK, mtDNA exit) are required for the message they want to deliver.

Minor points:

Abstract:
Opens (open?)
Introduction: This upregulate... (This upregulates?)
Results:
Please check the sentence: "That a delay exists before lipin-2 levels start to significantly increase after TLR activation is suggestive of the existence of a factor that is produced prior to and intermediates in the process."
Please check the sentence: "These results indicate that both, basal and stimulated IFN expression levels impact on lipin-2 ones."
INF-ß (IFN-ß!)
"In addition to inducing the formation of ISGF3, it has been reported that IFN-β may promote
the dimerization of STAT1 (GAF)" (GAS?)
maturated (matured?)
proteolitically (proteolytically!)
Il-1 (IL-1!)
specially (especially?)
ciclosporin (cyclosporine!)

Referee #2:

In this paper, de Pablo et al. evaluate the regulatory mechanisms of lipin-2, a phosphatidic acid phosphatase encoded by the gene Lpin2, which is mutated in an autoinflammatory disorder known as Majeed syndrome. de Pablo et al demonstrate that the expression of lipin-2 in stimulated bone marrow derived macrophages (BMDM) is regulated by TLRs, IFNs, and STAT1 participation. Further, the authors assess the role of lipin-2 during viral replication and activation of inflammasome NLRP3. Finally, the authors identify a correlation between COVID-19 severity and Lpin2 expression levels. Although the provided data sufficiently support the presented conclusions, further revision is required before publication. Primarily, in evaluating the role of lipin-2 in controlling viral replication, the authors limit their investigation to mainly assessing murine CMV and utilize poly(I:C), a viral nucleic acid mimetic, to synthetically model and support broader claims regarding the role of lipin-2 during events of viral infection. While the paper is generally well written, various areas for improvement and recommendations should be considered. Upon addressing the numerous concerns listed below, the paper presents the potential for publication at EMBO Reports.

1) In evaluating the role of lipin-2 in minimizing inflammation during viral recognition, the authors measure IL-1β production by BMDMs treated with poly(I:C), a viral nucleic acid mimetic. Although the use of a synthetic analog may provide an adequate model to evaluate IL-1β production, the conclusions and suggestions presented in the section are insufficiently supported by this experimental scope. Specifically, the authors note "cells re-equip themselves to recognize better the viral infection." Further evaluation should be made to determine the appropriateness of the model and the limit to which interpretations can be made. Finally, the authors can consider replicating experiments performed using poly(I:C) in conditions subjected to other viral infections as a proof of concept to corroborate findings obtained utilizing the viral nucleic acid mimetic. This work would also supplement data presented in the previous section in which the potential role of lipin-2 in MCMV viral replication was determined.

2) Despite a similar conclusion presented for both, Figures 6E and 6F present contradictory evidence. When assessing changes

in expression levels of siLpin2 conditions transfected with poly(I:C), Figure 6E displays an upregulation of Il-18 protein levels via ELISA. In contrast, Figure 6F lacks a significant difference in Il18 mRNA levels between siControl and siLpin2 conditions. Conclusions derived from data should be revised to avoid making general claims of "augmented synthesis" and to consider the possibility that different regulatory mechanisms may exist at the protein and mRNA levels.

3) Overall, numerous concerns exist surrounding statistical analyses of various figures. Comments and their corresponding figure are listed below.

a. In Figure 1A, the authors utilize multiple student's t-tests across various experimental groups. The authors should consider using a one-way ANOVA with the appropriate post hoc test (Dunnett's) to maintain more statistical power than multiple t-tests. Additionally, the figure legend of 1E lacks an explicit description of the significance associated with the "#" symbol. In Figure 4A, plots of mRNA expression levels for Lpin3, Rsad2, and Irf7 lack error bars, which further raises concerns regarding the use of biological or technical replicates.
b. The figure legend for Figure 3A states that the significance evaluated between IFN- β treated vs control cells however the data being presented in 3A evaluates IFN-γ conditions. Additional confirmation should be performed to verify that these levels of significance apply to the correct experiment.
c. Peak 1 in Figure 3C, presents data in which both IFN- β and IFN-γ conditions are statistically significant ($p<0.001$) despite their discrepancy in STAT1Chip-Seq reads. Visual comparison across the other bar graphs raises uncertainty regarding the accuracy of assigning statistical significance especially if comparisons were performed relative to the control within each peak.
d. The figure legend of Figure 4A is absent of any explanation regarding biological or technical replicates used to generate numerous plots evaluating mRNA fold change across Lpin1-3 and several ISGs.
e. Similarly, numerous figures throughout the paper are absent of error bars despite the use of replicates, as stated in the figure legends, to generate the corresponding example. For example, this is observed in the plot of PI+ cells in Figure 6K.

4) Experiments assessing relative expression require accurate normalization otherwise conclusions drawn from these figures will remain limited. For example, relative phosphorylation of p-p38 presents differing initial starting points of siControl and siLpin2. Therefore, it is hard to determine whether observed increases are due to experimental conditions or inherently higher basal levels. Similarly, Figure 5H evaluates the relative expression of NLRP3. However, time 0 datapoints between siLpin2 and siControl are drastically different. Without proper normalization, it is unclear as to whether "increases" in NLRP3 are due to inherently higher basal expression levels or effects of poly(I:C) stimulation. Plots in Figure 6H raise similar concerns.

5) Numerous concerns arise regarding immunoblot analysis. These comments and their corresponding figures are listed below.

a. Figure 5H presents an immunoblot presenting p-ERK, p-JNK, and p-p38. Although the authors are primarily concerned with evaluating phosphorylated levels, they fail to include the additional control of total protein (unphosphorylated) levels. This is required to accurately depict and interpret relative levels of activation.
b. Figure 6H lacks a loading control. This limits interpretations as to whether changes in expression levels are of biological significance or due to potential technical errors.
c. Numerous immunoblots of Lipin-2 occasionally detect protein levels as a double band however this phenomenon is inconsistent throughout the figures. Inconsistent protein detection raises concern regarding the specificity of the antibody or execution of downstream quantification which may impact graphical representation. For example, the presence of a double band is apparent in Figure 1, most notably in Figure 1D.

6) For Figure 1D, the authors should consider evaluating poly(I:C)-treated Lipin-2 expression levels at a range of time points that mirrors the experimental conditions performed for LPS-treated BMDMs. An expanded range of time points is required since the experimental design is a time-course analysis evaluating a potential delay before observing increases in lipin-2 expression level. Interpretation is limited when only 3 time points are measured.

7) Subtle revision is required for the conclusion derived from Figure 8C. A correlation coefficient with a value of 0.534 presents a moderate strength of correlation. Interpretations should be revised to reflect this calculation.

The authors present novel regulatory activities of lipin-2 in controlling anti-inflammatory and antiviral functions. These findings can be insightful in elucidating the mechanisms of Majeed syndrome. Additionally, the authors identify Lipin2 as a potentially useful biomarker in predicting severity among COVID-19 patients. Due to the extensive participation of lipin-2 in driving the interferon response, the presented work is significant for the basis of future work.

Referee #3:

In the manuscript by de Pablo et al. "Lipin-2 Is a Master Regulator of the Antiviral and Anti-Inflammatory Responses to Interferon" the authors make an attempt to characterize the regulation of expression and the immune functions of lipin-2, a phosphatidic acid phosphatase, that is inducible by interferons (IFN). Functional characterization of novel interferon-stimulated genes (ISGs) is a very important topic in the context of infectious and autoimmune diseases, which makes this study potentially

interesting to a broad audience. However, the presented work is seriously technically flawed to a degree that makes it impossible to evaluate the validity of the conclusions drawn by the authors. I summarized the main points below:

Major criticism:

1) In Fig 1, 2, S1 the authors rely on western blot analysis to assess lipin-2 expression in different conditions. However, I am not at all convinced that the antibody they are using is recognizing the lipin-2 protein as claimed. Any information about the lipin-2 specific antibody used is completely missing from the manuscript besides a single notion in the methods section "All other reagents were purchased from Sigma-Aldrich". The antibody used in the manuscript produces a signal corresponding to 140 kDa, whereas the predicted molecular weight of the lipin-2 protein is ~100 kDa and all commercially available antibodies I have encountered (including Sigma-Aldrich) produce a signal at ~80-110 kDa. A positive control (e.g. overexpressed lipin-2) is required to validate this antibody. Comparing the signal between the wt and lipin2-deficient cells is not helpful in this case as only a cropped blot is shown and there is no positive control. Without a proper validation of Ab specificity, it is impossible to judge whether the cells used in the study actually express lipin-2.

2) In Fig 1I the cells lacking the IFNAR1 still respond to IFN-beta (upregulation of lipin-2)! What is the explanation for that? This raises the question whether the IFNAR1 is indeed completely knocked out in these cells.

3) In all qPCR experiments: mRNA levels are shown/described in legend as "mRNA relative expression" - relative to what? The mRNA levels should be expressed as either absolute values (copies or Ct values per cell or per ng input RNA) or relative to a known housekeeping gene. The data in the figures do not allow to evaluate if the studied samples express biologically meaningful levels of lipin-2 mRNA both at the baseline and upon induction with IFNs.

4) In Fig 4 authors show a direct quantitative comparison of MCMV replication in wt BMDMs and lipin-2 -/- BMDMs (Fig 4B, C D) and animals (FIg 4E), however such quantitative comparison is incorrect, because these are two different cell lines / mouse strains. As lipin-2 is important in lipid metabolism, its knockout might have a pleiotropic effect (e.g. completely altering the metabolism of the corresponding animals and baseline expression of many genes), which does not allow to unequivocally interpret the enhanced viral replication in the lipn-2 knockouts compared to the wild type. Such experiments aiming to prove the direct link between viral replication and lipin-2 expression should be done using either specific inhibition of lipin-2 (e.g. pharmacologically or using siRNA) or overexpression of lipin-2 in the same cell line / mouse strain

5) In Fig 5, 6 and 7 authors perform lipin-2 siRNA knockdown experiments, however these experiments are not properly controlled - the untreated controls (no siRNA) are absent in all of the experiments. Moreover, the control siRNA (siControl) is not described anywhere in the manuscript. The effect of the siRNA on lipin-2 protein levels is not shown.

Other comments:

The manuscript should be edited for clarity.

REVIEWER #1

The authors have been investigating the role of lipin-2 - encoded by Lpin2 (LPIN2) - in inflammatory and immune responses in bone marrow derived macrophages (BMDMs) and in human monocyte-derived macrophages. Lipin-2 is a phosphatidic acid phosphatase, which plays a central role in lipid metabolism. Its regulatory role in inflammation became apparent in a disease known as Majeed syndrome, which results from LPIN2 mutation and is characterized by recurrent flares of fever and inflammation in their joints and skin.

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The authors subsequently addressed the role of lipin-2 in the replication control of murine CMV, which is known to be IFN-dependent. For practical reasons, a murine CMV carrying the GFP gene was used for the infection of wildtype and lipin-2 deficient BMDMs. Macrophage lipin-2 deficiency caused a significant increase in fluorescence forming units in vitro. In addition, mice deficient lipin-2 had a higher viral load in liver and spleen. Importantly, lipin-2 had no effect in the control of vaccinia virus, which is IFN-independent.

Using the TLR3 ligand poly I:C as a viral nucleic acid mimetic and the P2X7 agonist ATP, the authors examined the role of lipin-2 in inflammasome activation including the release of mature bioactive IL-1ß. As expected, lipin-2-deficient BMDMs produced higher levels of IL-1ß. LPIN2 knockdown using RNA interference in primary human macrophages also increased IL-1ß, although the effect was modest. MAPK activation was accelerated in lipin-2-deficient macrophages and, consistent with current knowledge, IL-1ß production was found to mainly depend on MAPK p38 (PMID: 19740675). Moreover, the absence of lipin-2 results in an increase in NLRP3 and CASP1 (expression and activity) as well as of the TLR3 itself and certain immunostimulatory genes (ISGs). Enhanced inflammasome activation resulted in an increase in pyroptosis, an inflammatory form of cell death that depends on inflammasome activation. The authors examine the role of mtDNA release to the cytosol and the production of ROS during inflammasome activation and overproduction of IL-1ß in the absence of lipin-2.

Last but not least, the authors used publicly available databases of gene expression arrays to evaluate

whether LPIN2 levels were altered in COVID-19 patients. They made the intriguing obeservation that patients with low LPIN2 had severe symptoms and were not able to recover from COVID19.

Comments

This is a well-written, detailed and important study based on modern and sophisticated methods.

The role of STAT1 was established in gene array experiments. No inhibitors were tested. Is this due to the limited availability of inhibitors of this pathway? The authors may comment on this.

We are not aware of any specific inhibitor for STAT1. All available inhibitors inhibit other STAT members or other targets. However, we have presented data using STAT1 knockouts (Fig. 3A), which allowed us to implicate STAT1 in *Lpin2* upregulation.

Why was IFN-γ used in the gene array experiments? IFN-ß? TLR agonists?

We used the arrays available at GEO databases using control and STAT1-deficient cells stimulated with IFN-γ. We could not find any arrays performed with STAT1–deficient cells that used IFN-β or TLR agonists.

The text referring to Figure 5 requires correction. The authors do not refer to Fig. 5A, which shows BMDM data, at all in the text! The authors then state: "the same behavior was also found in primary human macrophages silenced for LPIN2 (Fig. 5B and C)." However, according to the legend to Figure 5, BMDM data are shown in Fig. 5B, while data from primary human macrophages are shown in Fig. 5C.

Yes, our mistake. We apologize for the confusion. Now, we refer to Fig 5A in the text of the revised version (page 7, last paragraph, line 9).

LPIN2 silencing in primary human macrophages (Fig. 5C) is not impressive. However, this is not surprising. RNA interference is difficult (if not impossible) in primary human macrophages. This may be due to the innate features of macrophages, which may respond to siRNA sequence-dependent delivery vehicles and even the RNAi process itself (PMID: 28386261) or to the "digestive" capacity of this cell type. In many human macrophage studies, RNA interference has therefore been performed in the THP-1 cell line.

While we generally agree with the Reviewer's view, we would like to note that in the experiments depicted in Fig. 5C we were able to achieve a 70% reduction in *LPIN2* expression. Maybe not very impressive but certainly not modest either. We have tried silencing experiments in THP-1 cells as well, but the efficiencies have always been below those observed for human primary macrophages. To make these results more relevant we have included in the revised version of the manuscript an immunoblot showing the reduction of lipin-2 protein in the silenced human macrophages (New Extended View Figure 1). This should make a more convincing case.

If human primary macrophages would play a more significant role in this work, which is currently not the case, a minimal phenotype would be desirable (CD14, CD163, CD206), also because there are so many different protocols for macrophage generation (adherence, magnetic isolation, {plus minus} M-CSF, human vs. bovine serum, duration of culture).

The protocol that we use is based on the differentiation of human blood monocytes from buffy coats of healthy anonymous volunteer donors. Plastic-adhered monocytes were cultured during 10-14 days in RPMI 1640 with heat-inactivated 5% human serum, in the absence of exogenous cytokine mixtures. We have now explained this procedure with more detail in the revised manuscript (page 14, paragraph 4, line 3). Macrophages differentiated by this method express at least CD206, CD11b and DC-SIGN, and siRNAs are easily transfected by nucleofection (Municio C, et al. PLoS One. 2013 Apr 24;8(4): e62016).

What was the purpose of poly I:C transfection? A bit more explanation would be helpful.

Transfection of poly(I:C) is performed to introduce it inside the cell. In the cytosol, poly(I:C) can be recognized by inflammasomes and initiate caspase-1 activation, in accordance with previous work by others (Rajan et al. 2010, FEBS Lett.; 584: 4627–4632). We have now explained this with more detail in the revised manuscript (page 8, paragraph 2, line 3).

Overall, this work is an important contribution, which may however benefit from some streamlining measures. The authors might consider whether all data (MAPK, mtDNA exit) are required for the message they want to deliver.

Thank you for this frank assessment. As our intention is to present a picture as strong and complete as possible, we would rather prefer to keep all the information initially presented.

Minor points

Abstract: Opens (open?)

Introduction: This upregulate... (This upregulates?)

Results: Please check the sentence: "That a delay exists before lipin-2 levels start to significantly increase after TLR activation is suggestive of the existence of a factor that is produced prior to and intermediates in the process." Please check the sentence: "These results indicate that both, basal and stimulated IFN expression levels impact on lipin-2 ones." INF-ß (IFN-ß!). "In addition to inducing the formation of ISGF3, it has been reported that IFN-β may promote the dimerization of STAT1 (GAF)" (GAS?).
maturated (matured?) proteolitically (proteolytically!). Il-1 (IL-1!). specially (especially?). ciclosporin (cyclosporine!)

We have corrected these mistakes. It may seem confusing, but GAF is the STAT1 homodimer and GAS is the sequence where it binds.

REVIEWER #2

In this paper, de Pablo et al. evaluate the regulatory mechanisms of lipin-2, a phosphatidic acid phosphatase encoded by the gene Lpin2, which is mutated in an autoinflammatory disorder known as Majeed syndrome. de Pablo et al demonstrate that the expression of lipin-2 in stimulated bone marrow derived macrophages (BMDM) is regulated by TLRs, IFNs, and STAT1 participation. Further, the authors assess the role of lipin-2 during viral replication and activation of inflammasome NLRP3. Finally, the authors identify a correlation between COVID-19 severity and Lpin2 expression levels. Although the provided data sufficiently support the presented conclusions, further revision is required before publication. Primarily, in evaluating the role of lipin-2 in controlling viral replication, the authors limit their investigation to mainly assessing murine CMV and utilize poly(I:C), a viral nucleic acid mimetic, to synthetically model and support broader claims regarding the role of lipin-2 during events of viral infection. While the paper is generally well written, various areas for improvement and recommendations should be considered. Upon addressing the numerous concerns listed below, the paper presents the potential for publication at EMBO Reports.

1) In evaluating the role of lipin-2 in minimizing inflammation during viral recognition, the authors measure IL-1β production by BMDMs treated with poly(I:C), a viral nucleic acid mimetic. Although the use of a synthetic analog may provide an adequate model to evaluate IL-1β production, the conclusions and suggestions presented in the section are insufficiently supported by this experimental scope. Specifically, the authors note "cells re-equip themselves to recognize better the viral infection." Further evaluation should be made to determine the appropriateness of the model and the limit to which interpretations can be made. Finally, the authors can consider replicating experiments performed using poly(I:C) in conditions subjected to other viral infections as a proof of concept to corroborate findings obtained utilizing the viral nucleic acid mimetic. This work would also supplement data presented in the previous section in which the potential role of lipin-2 in MCMV viral replication was determined.

We thank the reviewer for these suggestions and ideas to improve our manuscript.

Regarding IL-1β production by actual viruses, we now present experiments performed in BMDMs and human macrophages infected with MCMV and HCMV. We show that the absence of lipin-2 increases IL-1β production in both cellular systems. We also present quantifications in serum from mice infected with MCMV that support the same role for lipin-2 *in vivo*.

The data are presented as a new figure EV1 and are described in Results (page 10, paragraph 2) and Materials and Methods (page 17, paragraph 3 and 4, line 7).

To improve clarity, we have revised the writing to eliminate potentially confusing sentences such as “generalized increased signaling” (page 8, paragraph 1, line 10) and "cells re-equip themselves to recognize better the viral infection" (page 8, paragraph 1, line 14).

2) Despite a similar conclusion presented for both, Figures 6E and 6F present contradictory evidence. When assessing changes in expression levels of siLpin2 conditions transfected with poly(I:C), Figure 6E displays an upregulation of Il-18 protein levels via ELISA. In contrast, Figure 6F lacks a significant

difference in Il18 mRNA levels between siControl and siLpin2 conditions. Conclusions derived from data should be revised to avoid making general claims of "augmented synthesis" and to consider the possibility that different regulatory mechanisms may exist at the protein and mRNA levels.

We now indicate in the revised manuscript that IL18 mRNA levels do not significantly change in cells (Gu et al., 1997). It is the conversion of pro-IL18 to the mature form that is used as a reliable marker for inflammasome activation (Lorden et al., 2017) (Results section: page 8, paragraph 3). Our interest was to use IL-18 production as another readout for inflammasome activation, in addition to IL-1β production. We showed that the mRNA level does not change in the absence of lipin-2, but IL-18 maturation is significantly increased. We have also removed the sentence utilizing the term "augmented synthesis". We agree with the Reviewer that it was potentially confusing.

3) Overall, numerous concerns exist surrounding statistical analyses of various figures. Comments and their corresponding figure are listed below.

a. In Figure 1A, the authors utilize multiple student's t-tests across various experimental groups. The authors should consider using a one-way ANOVA with the appropriate post hoc test (Dunnett's) to maintain more statistical power than multiple t-tests. Additionally, the figure legend of 1E lacks an explicit description of the significance associated with the "#" symbol. In Figure 4A, plots of mRNA expression levels for Lpin3, Rsad2, and Irf7 lack error bars, which further raises concerns regarding the use of biological or technical replicates.

We have performed one-way ANOVA with post hoc Holm-Sidak test in Fig. 1A. This is now clearly indicated it in the figure legend.

In the Figure 1 legend we have indicated that the # symbol stands for "Wt vs Ifnar$^{-/-}$ cells."

Regarding Figure 4A, these data came from microarray analyses published elsewhere (Blanc et al. 2013, Immunity, 38: 106-118). There were 25 time points in BMDMs infected with MCMV. Each time point (one microarray) was done with a unique biological replicate. This is the reason why the figure shows no error bars. We have now included this information in the figure caption. We also include an additional figure (new Figure 4B) showing *Lpin*2 mRNA levels in BMDMs infected with MCMV analyzed by qPCR. We have also included this information in the Results section (page 6, paragraph 3, line 8).

b. The figure legend for Figure 3A states that the significance evaluated between IFN- β treated vs control cells however the data being presented in 3A evaluates IFN-γ conditions. Additional confirmation should be performed to verify that these levels of significance apply to the correct experiment.

Thank you for pointing out this mistake to us. We have now corrected the figure legend. Additional confirmation was performed, as requested.

c. Peak 1 in Figure 3C, presents data in which both IFN- β and IFN-γ conditions are statistically significant ($p<0.001$) despite their discrepancy in STAT1Chip-Seq reads. Visual comparison across the other bar

graphs raises uncertainty regarding the accuracy of assigning statistical significance especially if comparisons were performed relative to the control within each peak.

We have revised the statistical analysis of this figure, and it was correct.

d. The figure legend of Figure 4A is absent of any explanation regarding biological or technical replicates used to generate numerous plots evaluating mRNA fold change across Lpin1-3 and several ISGs.

We have now corrected the legend to Figure 4A. As indicated above, these data come from microarray analyses published elsewhere (Blanc et al. 2013, Immunity, 38: 106-118). There were 25 time points in BMDMs infected with MCMV. Each time point (one microarray) was done with a unique biological replicate. This is the reason why the figure shows no error bars. We have now included this information in the figure caption. We also provide an additional figure (new Figure 4B) showing *Lpin*2 mRNA levels in BMDMs infected with MCMV analyzed by qPCR (biological triplicates).

e. Similarly, numerous figures throughout the paper are absent of error bars despite the use of replicates, as stated in the figure legends, to generate the corresponding example. For example, this is observed in the plot of PI+ cells in Figure 6K.

The error bars were too small to be appreciated in Figure 6K. We have redrawn the figure and corrected some inaccuracies in the figure legend. Also we have included the missing error bars in Figure 2A (Lpin3, Rsad2 and Ifr7). This was an unintentional omission when preparing the figure.

4) Experiments assessing relative expression require accurate normalization otherwise conclusions drawn from these figures will remain limited. For example, relative phosphorylation of p-p38 presents differing initial starting points of siControl and siLpin2. Therefore, it is hard to determine whether observed increases are due to experimental conditions or inherently higher basal levels. Similarly, Figure 5H evaluates the relative expression of NLRP3. However, time 0 datapoints between siLpin2 and siControl are drastically different. Without proper normalization, it is unclear as to whether "increases" in NLRP3 are due to inherently higher basal expression levels or effects of poly(I:C) stimulation. Plots in Figure 6H raise similar concerns.

While we appreciate the Reviewer's point of view on this matter, we would like to respectfully note that we presented the data in a way that allows the reader to appreciate both the changes in basal levels and those due to activation.

If we normalize the data to show the same level of expression/phosphorylation in both phenotypes of cells at time 0 as suggested by the Reviewer, we would just be showing the changes produced under the activation conditions. We would be missing the levels of expression/phosphorylation of these proteins in cells in the unstimulated state. We believe that basal information is important since lipin-2-deficient cells show increased levels of key receptors and signaling molecules. This is the case for NLRP3, for example. By showing the basal levels of those molecules we stress that the cells appear to be primed. We hope the Reviewer accepts our position in this particular point.

5) Numerous concerns arise regarding immunoblot analysis. These comments and their corresponding figures are listed below.

a. Figure 5H presents an immunoblot presenting p-ERK, p-JNK, and p-p38. Although the authors are primarily concerned with evaluating phosphorylated levels, they fail to include the additional control of total protein (unphosphorylated) levels. This is required to accurately depict and interpret relative levels of activation.

We have now included additional immunoblots showing the unphosphorylated kinases, as requested.

b. Figure 6H lacks a loading control. This limits interpretations as to whether changes in expression levels are of biological significance or due to potential technical errors.

The protein analyzed in Figure 6H was from cellular supernatants from the same number of cells. This is the control of the experiment. We now state this clearly in the corresponding caption. We are not aware of any protein that could be used as a loading control that is secreted and remains unchanged along the time-course of the experiment. In the field of inflammasome investigation this is an usual way of showing released inflammasome components and matured IL-1β.

c. Numerous immunoblots of Lipin-2 occasionally detect protein levels as a double band however this phenomenon is inconsistent throughout the figures. Inconsistent protein detection raises concern regarding the specificity of the antibody or execution of downstream quantification which may impact graphical representation. For example, the presence of a double band is apparent in Figure 1, most notably in Figure 1D.

We provide a new immunoblot for Figure 1D where lipin-2 is detected as a single band.

Regarding this inconsistency, we would like to note that the occasional detection of lipin-2 as a double band in PAGE analysis has also been reported by others (Sembongi et al JBC 2013, 288:34502-34513; Gimsey et al, JBC 2008, 283:29166-29174). The reason for this is unclear. It has been speculated to be related to differential phosphorylation states or other posttranslational modifications.

6) For Figure 1D, the authors should consider evaluating poly(I:C)-treated Lipin-2 expression levels at a range of time points that mirrors the experimental conditions performed for LPS-treated BMDMs. An expanded range of time points is required since the experimental design is a time-course analysis evaluating a potential delay before observing increases in lipin-2 expression level. Interpretation is limited when only 3 time points are measured.

We have modified Figure 1D to expand the time range, as suggested.

7) Subtle revision is required for the conclusion derived from Figure 8C. A correlation coefficient with a value of 0.534 presents a moderate strength of correlation. Interpretations should be revised to reflect this calculation.

Agreed. We have revised our interpretation and corrected the text accordingly (page 11, paragraph 1, line 3).

The authors present novel regulatory activities of lipin-2 in controlling anti-inflammatory and antiviral functions. These findings can be insightful in elucidating the mechanisms of Majeed syndrome. Additionally, the authors identify Lipin2 as a potentially useful biomarker in predicting severity among COVID-19 patients. Due to the extensive participation of lipin-2 in driving the interferon response, the presented work is significant for the basis of future work.

We thank the Reviewer for the positive assessment of our work.

REVIEWER #3

In the manuscript by de Pablo et al. "Lipin-2 Is a Master Regulator of the Antiviral and Anti-Inflammatory Responses to Interferon" the authors make an attempt to characterize the regulation of expression and the immune functions of lipin-2, a phosphatidic acid phosphatase, that is inducible by interferons (IFN). Functional characterization of novel interferon-stimulated genes (ISGs) is a very important topic in the context of infectious and autoimmune diseases, which makes this study potentially interesting to a broad audience. However, the presented work is seriously technically flawed to a degree that makes it impossible to evaluate the validity of the conclusions drawn by the authors. I summarized the main points below:

Major criticism:

1) In Fig 1, 2, S1 the authors rely on western blot analysis to assess lipin-2 expression in different conditions. However, I am not at all convinced that the antibody they are using is recognizing the lipin-2 protein as claimed. Any information about the lipin-2 specific antibody used is completely missing from the manuscript besides a single notion in the methods section "All other reagents were purchased from Sigma-Aldrich". The antibody used in the manuscript produces a signal corresponding to 140 kDa, whereas the predicted molecular weight of the lipin-2 protein is ~100 kDa and all commercially available antibodies I have encountered (including Sigma-Aldrich) produce a signal at ~80-110 kDa. A positive control (e.g. overexpressed lipin-2) is required to validate this antibody. Comparing the signal between the wt and lipin2-deficient cells is not helpful in this case as only a cropped blot is shown and there is no positive control. Without a proper validation of Ab specificity, it is impossible to judge whether the cells used in the study actually express lipin-2.

We thank the Reviewer for pointing out this important aspect of our study. We must apologize for not having clearly indicated the source of the antibody used in our study. Our lipin-2 antibody was from Bethyl Laboratories (A303-703A). This antibody has been previously used by others in murine macrophage cell lines, and the protein also runs close to 140-kDa (Watahiki et al., 2020, BBRC, 524: 477-483). The authors have also shown that the antibody recognizes overexpressed lipin-2 in 293T cells (Watahiki et al, 2021, 22: 2893). Lipin-2 is heavily phosphorylated in cells. This radically changes its electrophoretic mobility from a predicted 95 kDa to the observed ~140-150 kDa band in SDS-PAGE (Eaton et al. 2014, JBC, 289: 18055-16066). We acknowledge this in the revised manuscript (page 4, paragraph 3, lines 5). We have also included the source of the antibody in Materials and Methods (page 13, paragraph 4, line 8).

In addition, we have analyzed samples from lipin-2-overexpressing HEK-293 cells and a tagged construct lipin-2-EGFP by immunoblot. The antibody used in our work recognizes overexpressed lipin-2. We show also that the construct GFP-lipin-2 is also recognized by an anti-GFP-antibody. We have now included this information in Supplemental Material (New Appendix Figure S2) and commented on it in Results (page 4, paragraph 3, line 12).

2) In Fig 1I the cells lacking the IFNAR1 still respond to IFN-beta (upregulation of lipin-2)! What is the explanation for that? This raises the question whether the IFNAR1 is indeed completely knocked out in these cells.

K.O. animals for IFNAR1 were from Matthew Albert's laboratory and have been well described in the literature (Schilte et al., J.Exp. Med. 2010, 207: 429-442). Thus it is to be expected that BMDMs from these animals do not express IFNAR1. We would like to indicate that lipin-2 levels in the IFNAR1-deficient cells are exceedingly low (a lesser exposed gel would have failed to show a band). It is possible that the second chain of the type I IFN receptor (IFNAR2), that is still present in IFNAR1 KO cells, could minimally participate in lipin-2 upregulation. It has been described that both murine and human homodimers of IFNAR2 can phosphorylate STAT1 and STAT2 (Zoellner et al, 2022, Front. Microbiol. 13: 947169).

3) In all qPCR experiments: mRNA levels are shown/described in legend as "mRNA relative expression" - relative to what? The mRNA levels should be expressed as either absolute values (copies or Ct values per cell or per ng input RNA) or relative to a known housekeeping gene. The data in the figures do not allow to evaluate if the studied samples express biologically meaningful levels of lipin-2 mRNA both at the baseline and upon induction with IFNs.

The reference genes (housekeeping genes) used for qPCR in this study were *Gapdh* for murine and *ACTB* for human cells, as indicated in Materials and Methods (Quantitative PCR) section. We have also included this information in the figure legends.

4) In Fig 4 authors show a direct quantitative comparison of MCMV replication in wt BMDMs and lipin-2 -/- BMDMs (Fig 4B, C D) and animals (FIg 4E), however such quantitative comparison is incorrect, because these are two different cell lines / mouse strains. As lipin-2 is important in lipid metabolism, its knockout might have a pleiotropic effect (e.g. completely altering the metabolism of the corresponding animals and baseline expression of many genes), which does not allow to unequivocally interpret the enhanced viral replication in the lipn-2 knockouts compared to the wild type. Such experiments aiming to prove the direct link between viral replication and lipin-2 expression should be done using either specific inhibition of lipin-2 (e.g. pharmacologically or using siRNA) or overexpression of lipin-2 in the same cell line / mouse strain.

Thank you for the insight. We are not aware of any specific inhibitors for lipin-2. We have performed experiments using siRNAs, as suggested. The new Figure 4F shows that MCMV replicates more in lipin-2-silenced macrophages. We have included siControl cells and non-transfected/non-silenced cells, as requested as well by the Reviewer in Comment #5, below. We have included this information in the Results section (page 7, paragraph 2).

5) In Fig 5, 6 and 7 authors perform lipin-2 siRNA knockdown experiments, however these experiments are not properly controlled - the untreated controls (no siRNA) are absent in all of the experiments. Moreover, the control siRNA (siControl) is not described anywhere in the manuscript. The effect of the siRNA on lipin-2 protein levels is not shown.

We have included the information regarding negative controls for siRNA knockdown experiments in Materials and Methods (page 15, paragraph 1 and 2). We apologize for this omission.

Now we provide immunoblots showing lipin-2 expression in silenced cells (New Fig. 4F for RAW264.7 cells, and new Fig. EV1C for human macrophages).

We also provide data using untreated controls that we refer to as 'non-silenced cells' in silencing experiments performed in RAW264.7 cells (New Fig. 4F, new Fig. 6C and new Appendix Figure S8). We show that non-silenced cells and cells treated with siControl have similar responses regarding MCMV infection, *Il1b* expression after poly(I:C) stimulation and IL-1β production after inflammasome activation.

Other comments:

The manuscript should be edited for clarity.

We have enlisted the help of a native English speaker who assisted us in improving clarity.

Dear Prof. Balboa,

Thank you for the submission of your revised manuscript to our editorial offices. I have already forwarded the reports from the three referees that I asked to re-evaluate the study, you will find again below. As you know, referees #1 and #2 are satisfied by the revisions and think that the manuscript should be published in EMBO reports. In contrast, referee #3 indicates remaining issues and states that the manuscript requires further revisions. After going through your preliminary point-by-point response, and also after cross-commenting with the other referees, I ask you to address the remining points of referee #3 in a final revised manuscript as indicated in your letter. Please also provide a final p-b-p-response for these.

Moreover, I have these editorial requests:

- Please reduce the number of keywords to 5.

- 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 (for main, EV and Appendix figures) of the final revised manuscript. 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. 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. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

- Please make sure that all figure panels are called out separately and sequentially. Presently, it seem a callout for Fig. 8B is missing. Please check.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. It seem presently grants SAF2016-80883-R and MICIN/AEI/10.13039/501100011033 have not been entered into the submission system.

- Please provide all the materials and methods information in the main manuscript text file. Thus, please move that part from the Appendix to the main text.

- Please add scale bars of similar style and thickness to all the microscopic images, 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.

- Finally, please find also 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 comments. Please use the attached file as basis for further revisions and 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 (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) 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.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

All points I have raised have been sufficiently addressed. Especially, the new immunoblot showing the reduction of lipin-2 protein in the silenced human macrophages is convincing.

I have no further comments.

Referee #2:

In the revised version of "Lipin-2 Is a Master Regulator of the Antiviral and Anti-Inflammatory Responses," de Pablo et al. appropriately addressed previous comments and concerns. In experiments measuring IL-1B production by poly(I:C)-treated BMDMs, the authors have now extended their findings to include data from MCMV- and HCMV-infected macrophages. These additional findings support original results utilizing the viral nucleic acid mimetic, poly(I:C). Therefore, the conclusions presented are better supported. Previous concerns regarding statistical tests have been addressed primarily through the revision of original inaccuracies. Similarly, general concerns surrounding confusing or contradictory figure legends have now been addressed.

Regarding western blot analyses, the authors have provided improved immunoblots addressing concerns included in the initial review. For example, these revisions now include immunoblots of unphosphorylated kinases to allow unambiguous evaluation of relative levels of activation. Additionally, an immunoblot for the detection of lipin-2 as a single band has been included, which eliminates uncertainty of their detection methods. Although the authors have declined revisions regarding uniform normalization across basal and activated conditions, their justification is fine.

Overall, the conclusions presented in the paper are adequately supported by revised experiments and are further corroborated with additional experiments performed. Previous concerns regarding statistical tests and confusing figure legends across multiple experiments in the paper have now been addressed in the revised version.

Referee #3:

In the revised version of their manuscript authors performed additional experiments and revised parts of the text to address the reviewers' comments. In my opinion the manuscript has significantly improved both in terms of quality of the results and readability. However, several issues still remain requiring further revision:

In the point 2 of my review, I raised an issue of Lipin-2 being induced by IFN-beta in the IFNAR1 knockout cells, suggesting that the receptor knockout might be incomplete. I find the response provided by the authors not convincing. IFN-beta works by binding to IFNAR1/IFNAR2 heterodimer, therefore explaining the unexpected IFN-beta activity (lipin-2 induction) in these cells would require to postulate an alternative mechanism of type-I IFN signalling. Moreover the "Schilte et al." paper cited by the authors deals with synthetic chimeric receptors activated by artificial agonists and does not show that IFN-beta can signal in the absence of the IFNAR1 chain. The simplest explanation here is that the cells still express the IFNAR1 is some form. The knockout should be confirmed, or the corresponding data has to be removed entirely from the manuscript.

Other comments:

Fig 6L - IL-1b is very efficiently induced by poly(I:C) in the NLRP3 knockdown conditions in the wt cells (only marginally lower than control, compare the light blue bars), which suggests that NLRP3 role in that is either non-existent or completely redundant. The results from wt and KO cells cannot be directly quantitatively compared (see point 4). The conclusions (page 9) regarding the role of nlrp3 and nlrp3 inflammasome in the response to transfected poly(I:C) therefore should be revised.

In the same paragraph authors write "lipin-2 restrains NLRP3 inflammasome activation during recognition of cytosolic viral nucleic acids", but they do not actually use any viral nucleic acids in these experiments, please revise.

In Fig 7I, there is no difference in IL-1b induction by poly(I:C) in the presence or absence of cyclosporin A in wt animals, which does not support authors' conclusions that "both mtDNA release to the cytosol and oxidative events play a role during inflammasome activation by viral nucleic acid mimics". The conclusions should be revised.

In my opinion, the data provided in Fig. 6 and 7 is rather weak to support the conclusions drawn by the authors and should be moved to supplementary. The main text should be revised accordingly.

The manuscript title should be revised: it is not clear why Lipin-2 is called a "master regulator" in the title, since its expression itself is regulated by other genes (e.g. IFNs). Moreover, as the mechanism of action is not demonstrated, it is not clear if lipin-2 effects are direct or indirect.

Referee #3

In the revised version of their manuscript authors performed additional experiments and revised parts of the text to address the reviewers' comments. In my opinion the manuscript has significantly improved both in terms of quality of the results and readability. However, several issues still remain requiring further revision:

In the point 2 of my review, I raised an issue of Lipin-2 being induced by IFN-beta in the IFNAR1 knockout cells, suggesting that the receptor knockout might be incomplete. I find the response provided by the authors not convincing. IFN-beta works by binding to IFNAR1/IFNAR2 heterodimer, therefore explaining the unexpected IFN-beta activity (lipin-2 induction) in these cells would require to postulate an alternative mechanism of type-I IFN signalling. Moreover the "Schilte et al." paper cited by the authors deals with synthetic chimeric receptors activated by artificial agonists and does not show that IFN-beta can signal in the absence of the IFNAR1 chain. The simplest explanation here is that the cells still express the IFNAR1 in some form. The knockout should be confirmed, or the corresponding data has to be removed entirely from the manuscript.

We regret that Reviewer #3 finds our response not convincing. We would like to note that the IFNAR1 knockout cells were not made by us; they were provided by the original producers of the KO mouse. Please note in Fig. 1I that induction of the well decribed ISG viperin via TLR3, TLR4 receptor activation or IFN-β stimulation is completely abrogated in the IFNAR1 knockout cells. This suggests that the receptor knockout is complete.

As for our studies regarding lipin-2, what we wanted to explore with these cells was whether the increased expression of lipin-2 during TLR4 and TLR3 stimulation was due, at least in part, to the production of IFN. We reasoned that the best manner to study this was by using cells deficient in the receptor for the cytokine. Our data clearly show that elimination of Ifnar1 (whether complete or not) strongly reduces the upregulation of lipin-2. As a positive control we used also IFN-β. The findings support the view that IFN participates in lipin-2 upregulation during TLR3 and TLR4 stimulation. The upregulation of lipin-2 by IFN was also very strongly decreased by the elimination of Ifnar1.

We tried to be as transparent as possible about these results in our manuscript. Thus we tempered our conclusions accordingly. We state: “cells deficient in the IFN-β receptor subunit 1 (*Ifnar1*) displayed reduced expression levels of lipin-2, and their capacity to increase lipin-2 levels after TLR activation or IFN-β treatment was significantly reduced.” (Page 5, first paragraph).

Other comments:

Fig 6L - IL-1b is very efficiently induced by poly(I:C) in the NLRP3 knockdown conditions in the wt cells (only marginally lower than control, compare the light blue bars), which suggests that NLRP3 role in that is either non-existent or completely redundant. The results from wt and KO cells cannot be directly quantitatively compared (see point 4). The

conclusions (page 9) regarding the role of nlrp3 and nlrp3 inflammasome in the response to transfected poly(I:C) therefore should be revised.

In accordance with the reviewer's opinion, we have revised our conclusions. We now state: "Together, these data demonstrate that lipin-2 restrains NLRP3 inflammasome activation during recognition of cytosolic viral nucleic acids mimics". (Page 9, last paragraph).

In the same paragraph authors write "lipin-2 restrains NLRP3 inflammasome activation during recognition of cytosolic viral nucleic acids", but they do not actually use any viral nucleic acids in these experiments, please revise.

Yes, the reviewer is correct. We have revised this sentence as follows: "lipin-2 restrains NLRP3 inflammasome activation during recognition of cytosolic viral nucleic acid mimics" (Page 9, first paragraph).

In Fig 7I, there is no difference in IL-1b induction by poly(I:C) in the presence or absence of cyclosporin A in wt animals, which does not support authors' conclusions that "both mtDNA release to the cytosol and oxidative events play a role during inflammasome activation by viral nucleic acid mimics". The conclusions should be revised.

We have replaced that sentence as follows: "Overall, these results suggest that lipin-2 restrains both mtDNA release and oxidative events that play a role during inflammasome activation by viral nucleic acid mimics". (Page 9, last paragraph).

In my opinion, the data provided in Fig. 6 and 7 is rather weak to support the conclusions drawn by the authors and should be moved to supplementary. The main text should be revised accordingly.

We do not understand this comment. Whether in the main text or in Supplementary, the data would be shown anyway. So, what difference does it make to move these figures to Supplemental? The data and conclusions would remain the same.

The manuscript title should be revised: it is not clear why Lipin-2 is called a "master regulator" in the title, since its expression itself is regulated by other genes (e.g. IFNs). Moreover, as the mechanism of action is not demonstrated, it is not clear if lipin-2 effects are direct or indirect.

We have revised the title to eliminate the "master regulator part". The title now states that lipin-2 regulates the antiviral and anti-inflammatory responses to interferon.

Prof. María Balboa
Spanish National Research Council (CSIC)
Institute of Molecular Biology and Genetics (IBGM)
Calle Sanz y Fores 3
Valladolid 47003
Spain

Dear Prof. Balboa,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Reporting Checklist for Life Science Articles (updated January

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

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
- 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

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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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.	**Yes**	Materials and Methods
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	All animlas included
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 legends, Materials and Methods

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	

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Ethics	**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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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	
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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.

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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)
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If publicly available data were reused, provide the respective **data citations in the reference list**.	**Yes**	Data sets cited and accesion numbers provided