EMBO reports

RNA Sensor LGP2 inhibits TRAF Ubiquitin Ligase to Negatively Regulate Innate Immune Signaling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 10 October 2017

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

As you will see, all three referees highlight the general interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here. Most importantly though, all referees point out that the physiological and functional relevance of the findings needs to be strengthened, and that interactions need to be proven assaying also endogenous proteins. Also the claim that the described function of LPG2 is MAVS-independent needs to be supported with further data (point 5 of ref. #2 and second specific point of ref. #3). Finally, referees #2 and #3 also indicate that several control figures or control experiments are missing, which needs to be addressed.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can

submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors: http://embor.embopress.org/authorguide#manuscriptpreparation

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable.

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

Please also provide an ORCID for the co-corresponding author and link it to the EMBO reports profile.

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.

REFEREE REPORTS

Referee #1:

Parisien et al have analyzed the roles of a RIG-I-like receptor family protein, LGP2. LGP2 expression inhibited virus-induced antiviral gene expression, MAVS signaling, activation of NF-kB and IRF-3. This inhibitory activities were also confirmed by the data from LGP2-deficient mice. LGP2 was associated with TRAFs and inhibited MAVS- or TRAF-induced NF-kB activation. This inhibition was retained in a mutant LGP2 lacking both RNA binding and ATPase activities. The data further showed LGP2 inhibited TRAF ubiquitin ligase activity.
Overall experiments are well performed and the results are clear, novel and interesting. This reviewer, however, has several concerns to be addressed as follows.

1. Association of LGP2 with TRAFs is analyzed only in overexpression experiments. It should be also tested whether endogenous LGP2 is associated with endogeneous TRAFs.

2. Inhibition of TRAF ubiquitin ligase activity by LGP2 is also shown only in overexpression experiments. How about the ubiquitin ligase activity of anti-TRAF immunoprecipitates from LGP2-deficient cells? It should be tested whether the activity is enhanced or not in the absence of LGP2.

Referee #2:

In their manuscript the authors describe the RIG-I like receptor LGP2 as a negative regulator of innate immune signaling. LGP2 overexpression in HEK293 cells inhibits innate immune signaling in response to Sendai Virus infection (readouts: IFNb, IRF3 and NFkB promoter induced luciferase activity and mRNA expression of antiviral genes) while antiviral responses appear enhanced and more rapid in absence of LGP2 (Mefs LGP2 -/-, HEK293 LGP2 KO). LGP2 overexpression inhibits

antiviral signaling induced by overexpression of MAVS or TRAF proteins, demonstrating that the inhibition occurs downstream of MAVS and possibly at the level of TRAF proteins. Indeed, by immunoprecipitation of TRAFs the authors identify an interaction of LGP2 with the C-terminal MATH domain of TRAF2, 3, 5 and 6 which does not require the RNA binding or ATP hydrolysis activities of LGP2. Mechanistically LGP2 interferes with the ubiquitin ligase activity function of TRAF proteins, which is required for functional innate immune signaling induced by cytokines like IL1-b and TNFa or by activation of MAVS and STING signaling.

Overall Opinion:
In their study the authors approach a very interesting and yet enigmatic topic: the role of the RIG-I like receptor LGP2 in innate immune signaling. Quality of the data is not satisfying, as they lack several controls and should be supported with alternative approaches. Still, this work would be very valuable to the field since the function of LGP2 is unclear and controversially discussed. However, the study, in its current form, is not convincing and requires further experimental work supporting the authors' conclusions with more physiological approaches and addressing some open questions.

Major Comments:

Major concern:
1. The authors' conclusions are mainly derived from very unphysiological experimental settings involving co-transfection of two or even three plasmids, two of which are expression plasmids. In this experimental setup induction of ER stress is a huge concern which would shut down protein expression and could be a main reason for the observed reduced (reporter) gene expression in LGP2 overexpressing cells. Since major conclusions are based on immunoprecipitation experiments which are performed with overexpression of TRAFs and LGP2 these interactions should be confirmed by reverse IPs and supported by microscopy/colocalization analysis.
Some key experimental controls appear to be missing from figures within the manuscript. For example:
a. All Figures: Detailed statements regarding replicate numbers (biological and technical) and information on statistical analysis should be included. Are data derived from independent experiments?
b. Fig 1A-F: LGP2 overexpression might cause ER stress which would lead to translational shutdown and could cause unspecific effects. An unrelated expression vector would be a better control than an empty vector. Since cellular stress could reduce SeV replication/infection efficiency, which might contribute to the observed effects, viral replication efficiency should be controlled for. Another control would be a replication independent stimulus like poly(I:C). Scales for D and E should be adjusted.
c. Fig 1G-J: SeV replication and infection efficiency should be measured in WT and LGP2 KO Mefs since slightly enhanced viral PAMP amounts could account for the minor changes (2fold increase) measured for transcript level. In addition an alternative stimulus (different virus or poly(I:C)) would serve to support the authors' conclusions. A western blot demonstrating absence of LGP2 protein in KO cells should be included.
d. Fig 2 and Fig 4: How did the authors control for increasing plasmid-DNA amounts due to LGP2 titration? Were plasmid-DNA amounts in samples containing low LGP2 plasmid concentrations adjusted with an unrelated expression plasmid to control for cellular stress caused by protein overexpression and presence of cytosolic DNA? Overall cotransfection of three plasmids, two of which are expression plasmids, appears very unphysiological and prone to cause cell stress which could underlie or contribute to the observed effects on promoter activities. Using an inducible expression system or stable cells lines appears more suitable. A promoter-reporter construct which is not affected by MAVS and LGP2 overexpression could serve as a control to exclude unspecific effects on reporter expression due to ER stress. More detailed information about the transfection procedure should be included in material/methods.
e. Fig 3: Better images with less background should be provided for all western blots. A statement regarding the number of biological replicates is missing. The replicates should be used to quantify IRF3 phosphorylation level and statistical analysis on IRF3 phosphorylation and NFkB activation upon SeV infection in presence and absence of LGP2.
f. Fig 5: Experimental details are missing. The statement "with or without LGP2 titration" does not indicate if and what kind of control plasmid was used to control for the effects of a) introduction of plasmid DNA into the cytosol and b) protein overexpression. Please provide information on the cell line used for these assays. Instead of focusing exclusively on reporter activities as readout, transcript

level of an endogenous gene should be included.
g. Fig 7C: In various samples RIG-I and MDA5 expression seem very low or absent which compromises their use as controls (e.g. TRAF2 pulldown).
h. Fig 8: The data look promising but should be supported by ubiquitin pulldown, blotting for TRAF in presence or absence of LGP2.
2. Overall more details on experimental replicates and statistical analysis are required.
3. Does the "earlier and hyperactive" response to virus infection observed in absence of LGP2 functionally translate into enhanced cellular resistance to virus replication?
4. For immunoprecipitation experiments a non-specific IgG should be used as control for non-specific binding which is a concern in the overexpression setting.
5. The observed interaction of LGP2 with all tested TRAF proteins raises the question if this interaction is of direct or indirect nature (e.g. via MAVS). In 2006 the investigating group published an interaction of LGP2 and MAVS (Komuro et al., 2006). Interaction of MAVS with TRAF C-terminal domain is also well established in the field. This could be addressed in MAVS knockout cells. It also raises the question if LGP2 overexpression would block an interaction of TRAF and MAVS and thereby negatively regulate immune signaling since both, MAVS and LGP2 seem to bind to the C-terminal domain of TRAFs.
6. Another question which can easily be addressed is if the proposed interaction of LGP2 and TRAFs is lost upon innate immune activation. This would indeed support a specific and functional interaction of LGP2 and TRAF (of direct or indirect nature).

Minor Comments:
1. Fig 3B: The authors should include a statement regarding the pIkBa S32 signal in mock treated LGP2 KO Mefs. Was this observed in all replicate experiments?
2. Fig 3C: If possible, a western blot demonstrating the absence of basal LGP2 (basal LGP2 is not visible in WT) should be provided.
3. Fig 3D: A time resolved analysis of IRF3 phosphorylation (e.g. 2, 3, 4, 5, 6h post SeV infection) would support the authors' conclusion on more rapid IRF3 phosphorylation in absence of LGP2. Measuring SeV replication and in addition using an alternative stimulus (poly(I:C)) would control for potential differences in SeV replication in WT and KO cells.
4. Fig 5B: Is the annotation 1-4 ug correct? In reporter gene experiments 4-500 ng of LGP2 plasmid were transfected. The authors should provide plasmid concentrations rather than total plasmid amounts.
5. Fig 6B: A HA-RIG-I control blot for the FLAG-IP is missing. Please provide a larger section of the HA blot which would include the kDa range of HA-RIG-I. The control gel for HA (Lysate) should be separated longer so that HA-RIG-I expression can be evaluated.
6. Fig 7B: Why is the basal luciferase activity in the TRAF3 setting (-/-/-) so high? Details on statistical analysis are missing. What does average refer to (mean, median?) I assume mean but this should be specified. RLR pulldown and coimmunoprecipitation of TRAFs would support the authors' statements.
7. Please specify WCEB (whole cell extract buffer) in material and methods. In general more details on experimental procedures should be provided.
8. Please correct the following sentence (results section, Novel means of LGP2 TRAF suppression): The TRAF interaction is specific for LGP2, as neither MDA5 nor RIG-I were found to co-precipitate with and TRAF tested (Fig. 7C).
9. Overstatement, Fig 7: What exactly proofs auto-ubiquitination of TRAF proteins? Could they be modified by another ubiquitin ligase which might be a target of LGP2?

Referee #3:

In this study Parisien et al., elucidate the mechanism by which LGP2 negatively regulates IFN and NF-kB signaling. Using overexpression assays and LGP2 -/- MEFs the authors show that SeV-induced IFNβ, ISG56 as well as TNF genes are inhibited by LGP2.
The authors confirm their findings in human cells by generating a CRISPR-KO LGP2 293T cell line. Using immunoprecipitation assays the authors show that LGP2 interacts with the C-terminus of TRAF family members (2,3,5,6) and interferes with their ubiquitin ligase activity. The authors further show that these effects are independent of RNA binding and ATPase hydrolysis.

This is a very interesting, well-written, well-executed study. In general the experiments are of high

quality and well controlled. There are, however, some minor suggestions that could help strengthen some of their experiments and their conclusions and most importantly the functional relevance.

Specific points:

- Figure 1G-J: for this experiment it would be useful to show the mRNA kinetics to better appreciate the increased mRNA expression of cytokines in LGP2 -/- cells. Would the authors expect to see at any time point a decrease of cytokine mRNA due to the positive regulatory roles of LGP2? Or these effects do not apply to SeV-dependent induction?

- The authors conclude that LGP2 negatively regulates the pathway independently of MAVS based on results obtained using TNF, IL-1b and STING stimulations, which do not signal through MAVS. This is fine, however in the RIG-I/MDA5 pathway, since LGP2 has previously been shown to act as positive regulator and could enhance MAVS filaments, it is important that the authors truly rule out MAVS involvement. Furthermore, since TRAFs are recruited to MAVS, the interaction of LGP2 with TRAFs could be indirect. To rule out any involvement of MAVS in binding of LGP2 to TRAFs (and ubiquitination of TRAFs), the authors could perform the TRAF immunoprecipitation experiments (as shown in Figure 7 and figure 8) in MAVS knockout cells (if not available, use knockdown of MAVS). This will show that the interactions are independent of MAVS (or MAVS complexes). If truly independent of MAVS, ubiquitination of TRAFs should not change in MAVS KO vs WT. Pull-down of endogenous TRAFs (instead of only over-expression) would also be desirable to furthers strengthen functional relevance.

Figure 6C the last panel the label is missing (I assume is GAPDH). Please add.

Figure 7 and 8, please add control blots for ubiquitin (anti-ubiquitin antibody).

Figure 7: negative control for immunoprecipitation in the absence of Flag-Traf constructs, but in the presence of RLR, as it was done in Figure 6.

Figure 8: here the authors demonstrate inhibition of TRAF ubiquitination by LGP2. To strengthen this conclusion, the authors should perform the same immunoprecipitation of TRAF proteins in LGP2 -/- cells. TRAF ubiquitination should be enhanced.

- Please add molecular weigh markers to all immunoblots.

1st Revision - authors' response 20 February 2018

Response to Referee Comments, EMBOR-2017-45176V1

Referee #1

We are grateful for the reviewer's comment that overall our experiments are well performed and the results are clear, novel and interesting. Two concerns were raised:

1.1. It should be also tested whether endogenous LGP2 is associated with endogenous TRAFs.
A: This rather straightforward request presented the greatest obstacle for us, and despite numerous and costly attempts to provide beautiful data we have come up short. There are several technical reasons we are unable to provide the requested experiment. LGP2 steady state protein levels are extremely low in many cultured cells, though the protein is highly induced by virus infection. This low endogenous level complicates endogenous co precipitation experiments but would be surmountable if good reagents were available to detect LGP2. We have worked with LGP2 for many years, and have produced milligrams of purified protein from recombinant baculovirus vectors (eg. Bruns 2014). We have immunized dozens of mice and rabbits with this protein and yet have been unsuccessful in generating a high-quality antiserum (though our antibodies made simultaneously for RIG-I and MDA5 are excellent). We conclude that LGP2 is very non-antigenic. Similar experience has been shared anecdotally by others in our small community (e.g., Michael Gale, Mehul Suthar), requiring the use of rather poor commercially available antisera. Even this antibody source has

changed between the time of submission and revision, and now we find it only weakly detects even the virus-induced LGP2 protein in some cells. This has led to great frustration trying to satisfactorily address the reviewers' concerns- which we agree with.

It is relevant to point out that during our revision period, another paper was published in EMBO Journal that described a conclusion about an unrelated activity of LGP2 (van der Veen et al., EMBO J. 2018 doi:10.15252/embj.201797479). I note that this paper also lacks any endogenous coprecipitation
data, and examination of the published peer review file reveals this was not even raised
as a concern during review. Instead, the authors were encouraged to use CRISPR targeting to genetically interrogate their conclusion.

We have taken a similar approach to demonstrate and reinforce the physiological and functional relevance of our findings, by using CRISPR targeted cells in virus and poly(I:C) -induced transcription
assays. The results clearly support our earlier conclusions regarding the impact of LGP2 on IRF3 and NFκB transcription, highlighting the negative regulatory role of LGP2 that is lost upon knockout,
and leading to functional enhancement of antiviral gene transcription. Similar results are obtained from virus infection and from dsRNA stimulation. Moreover, we demonstrate that LGP2 expression does not alter other signaling systems non-specifically, namely those induced by IFNα and IFNγ. These data are provided as Figure 2G and 2H, and the possibility of indirect interactions has been addressed in the text. I sincerely hope that in view of these results, and given the technical considerations described above, that the reviewer will be persuaded that many lines of experimentation in both over-expression and genetic deficiency have led us to the same conclusions about LGP2 negative function.

1.2. How about the ubiquitin ligase activity of anti-TRAF immunoprecipitates from LGP2-deficient cells?
A: We have carried out the requested experiments and see little difference in overall ub signals from TRAF immunoprecipitates irrespective of LGP2 deficiency. This likely reflects the low endogenous levels of LGP2 compared to TRAFs, and TRAF IPs have more unbound TRAF than bound. In an experiment now included as Figure 8C, we demonstrate that TRAF ubiquitin transfer is clearly blocked by LGP2 and LGP2-H, but not RIG-I or MDA5.

Referee #2

This reviewer appreciated our study of a very interesting and yet enigmatic topic, but had many major

and minor issues to resolve. We did our best to address them all.
2.1. The reviewer is concerned that expression of proteins induces ER stress that would nonspecifically shut down protein synthesis and give rise to reduced reporter gene expression and suggests that TRAF and LGP2 interactions should be confirmed by reverse IPs.
A: While our cytoplasmically localized proteins are unlikely to cause ER stress, we understand the concern. To alleviate this concern we have executed control experiments and also carried out the suggested reverse IPs in two ways (new Figure 7D and E). One uses the same experimental setup but switches the IP and western antibodies, the other switches the tags and repeats the original IP and western antibodies. Both experiments clearly confirmed the original contention, that LGP2 coprecipitates with TRAFs.

2.1a. Detailed statements regarding replicate numbers and statistical analysis should be included.
A: These details have been included in the figure legends and methods.

2.1b. An unrelated expression vector would be a better control than an empty vector. A replication independent stimulus like poly(I:C). Scales for D and E should be adjusted.
A: We demonstrate that GFP expression does not alter LGP2 activity (Expanded view 2A) and that poly(I:C) signaling is suppressed by LGP2 (Fig. 2A-C, 3F,3H); Scales have been adjusted as suggested to normalize all graphs (WT activity normalized to 1).

2.1c. A western blot demonstrating absence of LGP2 protein in KO cells should be included.
A: Due to the low detection of endogenous steady state LGP2 protein as cited above, RT-qPCR was used to demonstrate loss of LGP2 mRNA (Fig. 1G inset).

2.1.d. How did the authors control for increasing plasmid-DNA amounts due to LGP2 titration?
A: We routinely use sheared salmon sperm DNA as a carrier to make all transfected DNA amounts equal. Similar results are obtained from including either empty vectors or GFP plasmid to make up the DNA amounts (Expanded view 2A). These details have been added to the text.

2.1.d.2. A promoter-reporter construct which is not affected by MAVS and LGP2 overexpression could serve as a control
A: We provide evidence that IFNα and IFNγ (ISRE and GAS) reporter genes are not suppressed by LGP2 under the same expression conditions, ruling out nonspecific or stress-related artifacts. (Fig. 2G-H).

2.1.e. Fig 3: Better images with less background should be provided for all western blots.
A: We apologize that due to file type and/or color space usage, the images in the review document appeared very dark with high backgrounds. We both enlarged the images and clarified the file types for the revised version for clarification.

2.1.f. Fig 5: Experimental details are missing...
A: We have now included additional details in the text and figure legend to clarify the confusion.

2.1.g. Fig 7C: In various samples RIG-I and MDA5 expression seem very low or absent which compromises their use as controls (e.g. TRAF2 pulldown).
A: We apologize for the lack of clarity due to differential western transfer of large vs. small proteins.
We provide a new image with a more balanced transfer and blot image.

2.1.h. Fig 8: The data look promising but should be supported by ubiquitin pulldown, blotting for TRAF
in presence or absence of LGP2.
A: We attempted this experiment twice, but the high level of ubiquitination in TRAF-overexpressing cells results in messy blots that cannot be interpreted.

2.2. Overall more details on experimental replicates and statistical analysis are required.
A: We apologize for this omission and have added experimental details throughout.

3. Does the "earlier and hyperactive" response to virus infection observed in absence of LGP2 functionally translate into enhanced cellular resistance to virus replication?
A: In the absence of LGP2, we observe both earlier and more potent responses at the transcription factor level, and this translates into greater virus or poly(I:C) -induced transcriptional activity within the
onset of the antiviral response measured by transcriptional responses. This increased activity was apparently saturated in the time course of Sendai virus plaque assays, resulting in low differential virus replication. A future study will compare LGP2 effects on different viral and inflammation pathways

4. For immunoprecipitation experiments a non-specific IgG should be used as control for non-specific
binding which is a concern in the overexpression setting.
A: We now provide two types of reverse-IP as well as several examples of co-precipitation and interference that are mediated by LGP2 but not RIG-I or MDA5 (Fig. 7D-E). These highly reproducible results coupled with our dissection of the TRAF binding site that reveals many noninteracting
fragments, give high confidence in the coprecipitation of LGP2 and TRAF.

5. The observed interaction of LGP2 with all tested TRAF proteins raises the question if this interaction is of direct or indirect nature (e.g. via MAVS).....It also raises the question if LGP2

overexpression would block an interaction of TRAF and MAVS and thereby negatively regulate immune signaling since both, MAVS and LGP2 seem to bind to the C-terminal domain of TRAFs.
A: We are very interested in the dynamics of LGP2-MAVS-TRAF interactions but as more technological development is required to make a complete mechanistic study we feel this is outside the focus of the current manuscript. Nonetheless the reviewer's point is well taken and will be a hypothesis tested in the future. Meanwhile we have revised the text to reflect the more circumspect view that the observed co-precipitation may represent a direct or indirect interaction bridged by another protein. Could this protein be MAVS? We provide evidence that LGP2 can interfere with MAVS-independent TRAF signaling and a new in Expanded view 2C demonstrates LGP2 retains its ability to interfere with TRAF-dependent TNF signaling even after knocking down MAVS with siRNA.

6. Another question which can easily be addressed is if the proposed interaction of LGP2 and TRAFs
is lost upon innate immune activation.
A: This is an intriguing suggestion, but our experiments do not support attenuation of the co-IP following innate immune activation.

Minor Comments:

1. Fig 3B: The authors should include a statement regarding the pIkBa S32 signal in mock treated LGP2 KO Mefs. Was this observed in all replicate experiments?
A: We interpret this as a low level of basal activity in the KO MEFs. Our limited access to KO MEFs
has prevented a more thorough analysis, but we have taken advantage of our CRISPR KO to examine functional responses to endogenous gene targets, and data provided are thoroughly reproducible demonstrating effects on both IRF3 and NFκB target genes consistent with LGP2 negative regulation.

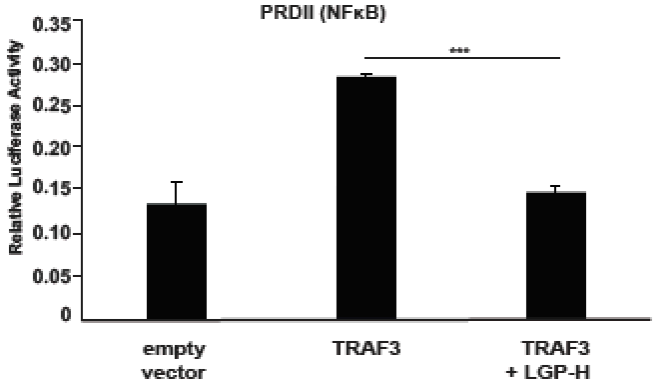
2. Fig 3C: If possible, a western blot demonstrating the absence of basal LGP2 (basal LGP2 is not visible in WT) should be provided.
A: As explained above, the low level and poor reagents for LGP2 make the detection of basal LGP2 challenging if not impossible. We used loss of virus-induced LGP2 as a proxy for CRISPR KO.

3. Fig 3D: A time resolved analysis of IRF3 phosphorylation (e.g. 2, 3, 4, 5, 6h post SeV infection) would support the authors' conclusion on more rapid IRF3 phosphorylation in absence of LGP2.
A: We provide the requested hourly time course in Expanded view 2B and the results support our initial conclusions.

4. Fig 5B: Is the annotation 1-4 ug correct? In reporter gene experiments 4-500 ng of LGP2 plasmid
were transfected. The authors should provide plasmid concentrations rather than total plasmid amounts.
A: These experiments were carried out in different size TC dishes, hence the different amounts of plasmid used for transfection. The text has been clarified.

5. Fig 6B: A HA-RIG-I control blot for the FLAG-IP is missing/include the kDa range of HA-RIG-I.
A: The RIG-I band was obscured in the original figure by a co-migrating nonspecific artifact band. This has been resolved on a new gel as suggested to clarify RIG-I.

6. Fig 7B: Why is the basal luciferase activity in the TRAF3 setting (-/-/-) so high?
A: This is a graphical treatment of data normalized to show side by side analysis. As TRAF3 activation of the reporter is low, the mathematical treatment raises the baseline. Importantly, we show that the TRAF3-induced activity is suppressed by LGP2-H returning it to baseline level. Here are the non-normalized data for TRAF3 and this will be included in the expanded view figure 2.



7. Please specify WCEB (whole cell extract buffer) in material and methods. In general more details on experimental procedures should be provided.
A: Again we regret any confusion and have expanded these details in the text.

8. Please correct the following sentence…
A: done

9. Overstatement, Fig 7: What exactly proofs auto-ubiquitination of TRAF proteins? Could they be modified by another ubiquitin ligase which might be a target of LGP2?
A: While the TRAF IP-western assay is typically used to reflect auto-ubiquitination, as we have not formally tested this, we have tried to edit the text to be more circumspect in the interpretation of observed Ub. It is TRAF-dependent and visible in a TRAF IP, creating slower migrating antibody reactive species above the position of TRAF. Additional data on TRAF mediated ubiquitination and specific interference is now provided in Figure 8C.

Referee #3

We are grateful to the reviewer for the comments that we conducted a very interesting, well-written, well-executed study of high quality and well controlled. Two minor points were raised:

3.1.a. It would be useful to show the mRNA kinetics to better appreciate the increased mRNA expression of cytokines in LGP2 -/- cells.
A: Thank you for this suggestion. We now provide time course mRNA data for both virus and poly(I:C) stimulation in the new Figure 3.

3.1.b. Would the authors expect to see at any time point a decrease of cytokine mRNA due to the positive regulatory roles of LGP2?
A: This is an interesting question, but please note that the positive regulatory role of LGP2 is specifically for MDA5 responses to long dsRNA, and irrelevant to RIG-I (Bruns et al, 2014). As Sendai virus infection triggers MAVS via RIG-I recognition of defective genomes, there is no reason to suspect LGP2 positive regulation. Our analysis confirms this to be the case.

3.2. …. it is important that the authors truly rule out MAVS involvement [in inhibition of TNF, etc.]….
A: The positive effects of LGP2 are not involved in Sendai virus recognition. To be sure that MAVS is not required to suppress TNF signaling to NFκB, we conducted an siRNA knockdown of MAVS followed by poly(I:C) or TNF stimulation with or without LGP2. MAVS knockdown inhibited poly(I:C) signaling as expected (nice control for the siRNA efficacy), but did not interfere with either TNF signaling or LGP2 suppression. This rules out a role for MAVS in LGP2 negative regulation. This is included in Expanded view 2C.

3.3 Figure 6C the last panel the label is missing (I assume is GAPDH). Please add.
A: done

Figure 7 and 8, please add control blots for ubiquitin (anti-ubiquitin antibody).
A: a new experiment was conducted to address this control. It is now included as Figure 8C

Figure 7: negative control for immunoprecipitation in the absence of Flag-Traf constructs, but in the presence of RLR, as it was done in Figure 6.
A: A new control experiment was conducted to address this comment. It is now included in Expanded view 2.

Figure 8: here the authors demonstrate inhibition of TRAF ubiquitination by LGP2. To strengthen this conclusion, the authors should perform the same immunoprecipitation of TRAF proteins in LGP2 -/- cells. TRAF ubiquitination should be enhanced.
A: We really like this suggestion and tried this experiment twice, but the high level of ubiquitination in TRAF-expressing cells results in messy blots that cannot be interpreted.

- *Please add molecular weight markers to all immunoblots.*
A: Done

2nd Editorial Decision 7 March 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, all referees now support the publication of your manuscript in EMBO reports.

Before we can proceed with formal acceptance, I have the following editorial requests:

Please format the references according to EMBO reports style (not more than 10 authors). See: http://embor.embopress.org/authorguide#referencesformat

The title presently contains many abbreviations. Could you come up with one with fewer abbreviations and without commas (but with not more than 100 characters including spaces)?

For the statistical analysis, can you please specify the test used to calculate p-values in the respective figure legends? Please also provide a paragraph in the methods section describing the statistics. See also: http://embor.embopress.org/authorguide#statisticalanalysis

The Western blot images sometimes differ very strongly in terms of contrast and brightness (background), even within one panel (see e.g. Fig. 6C or 7C). Could you provide in all Western panels as unmodified images possible, with similar background intensities? Further, some images are very grainy (e.g. in Fig. 3B), or show background textures (e.g. Fig. 8C). Do you have better quality images for these? However, if this represents the data as it looked on the film, we are happy to include the images as they are.

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

The labelling in Fig. 4 is rather small, and will be difficult to read in the online version. Could you provide this figure with bigger fonts, maybe not presenting the diagrams in landscape format? See also our guidelines for figure preparation:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

The blot WB:RLR in Figure 7C shows a thin vertical line between the TRAF5 and TRAF6 lanes. If the two blots are spliced from different experiments (films), please indicate this with a black line.

The same for the blot KO-GAPDH in Figure EV2B (between 3h and 4h). If this was spliced together, please indicate this by a black line.

What is the white horizontal line in the upper TRAF6 blot in Figure 8A?

Finally, please find attached a word file of the manuscript text with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address.

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study

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

REFEREEE REPORTS

Referee #1:

I understand the difficulty to analyze endogenous LGP2. My concerns have been addressed properly.

Referee #2:

The authors have conducted new experiments to clarify and extend their findings. The revisions to the paper fully address all of my previous comments and the paper is improved to the point that it should be highly attractive and relevant story for the innate immunity field.

Referee #3:

The authors have addressed my previous concerns.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Curt Horvath
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45176V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size for individual experiments carry at least 3 technical replicates and reflect behavior of minimal 3 biological replicates and typically >5.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293 cells (ATCC) and mouse embryonic fibroblasts (MEFs; gift of Dr. M. Gale Jr., University of Washington, Seattle, WA) were grown in DMEM supplemented with 10% cosmic calf serum (CCS, Hyclone) and 1% penicillin-streptomycin (Gibco-BRL). Cells are routinely tested for mycoplasma contamination and regularly restored from early passage frozen stocks. MEFs were used before the sixth passage.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No dual use concerns.