RNF123 has an E3 ligase-independent function in RIG-I like receptor-mediated antiviral signaling

Mr. Shuai Wang, Yong-Kang Yang, Tao Chen, Zhonghe Zhai and Danying Chen

Corresponding author: Danying Chen, Peking University

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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 06 January 2016

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in getting back to you, but we have only now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, referee 1 also points out several concerns and has a number of suggestions for how the study should be strengthened. The referee suggests to improve knockdown efficiencies (or use CRISPR-Cas9 to generate a knock-out) to potentially enhance the observed phenotype (point 3) and to analyze if a stronger overexpression of RNF123 results in a greater reduction of IFN induction (point 1). Moreover, he/she suggests to investigate what triggers the interaction between RNF123 and the RLR-CARD domain (point 5). Upon further discussion with the referees this point could be addressed by examining if the interaction increases upon virus infection or IFNβ stimulation or if RNF123 re-localizes within the cells under these conditions. All control experiments have to be provided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review.
REFEREE REPORTS

Referee #1:

In this study, Wang and colleagues identified the RING finger RNF123 protein as a novel negative regulator of the RLRs signaling pathway. The authors observe that RNF123 is able to reduce IFN induction upon RNA virus infection independently of its E3 ligase activity. They then show that RNF123 interacts with the CARD domain of RIGI and MDA5, and interferes with the formation of the RLR-MAVS mitochondrial complex. The study of the mechanisms that determine the outcome of the antiviral response is a very interesting topic of research, and the presented work can thus provide new knowledge in this field. Overall the data shown are consistent and the manuscript is well written. However, the physiological importance of RNF123 is not clear. The differences observed are moderate and the mechanistic part of the work is still preliminary. Specific issues are detailed below.

1. Western blots showing RNF123 protein levels should be included in Fig. 1. Upon overexpression of RNF123 (100ng) a maximum 50% reduction in IFN induction is observed. Would a higher amount of RNF123 result in stronger inhibition of the signaling?
2. Figure 1F is not convincing. In addition, RNF123 expression levels should also be shown in the WB.
3. SH-RNA-mediated knockdown efficiencies shown in Fig. 2 are very low. As a consequence, the phenotype observed is also very moderate. The authors should try to improve their KDs or to generate KO cell line by the CRISPR Cas9 technology. Reconstitution assays should also be performed to rule out off-target effects.
5. How RNF123 inhibits RLR-MAVS binding is still unclear. Is RNF123 binding to the RLRs CARD domain inducible? The authors should better investigate what triggers this interaction to better clarify RNF123 mechanism of action.
4. Where is RNF123 localized within the cell? The authors should provide imaging data showing its localization with respect to Rig-I, MDA5 and MAVS during infection. This information would be a nice complement to the current version of the manuscript.

Referee #2:

This manuscript describes the identification of RNF123 as a new negative regulator of the RIG-I/MDA5 antiviral signaling pathway. The authors show that overexpression of RNF123 decreases NF-kB-dependent gene induction and IFNbeta induction that occurs upon activation of RIG-I- and MDA5-dependent pathways. Conversely, knockdown of RNF123 has the opposite effect on these pathways, confirming that endogenous levels of RNF123 are functional in dampening antiviral signaling. Importantly, RNF123 overexpression or knockdown had corresponding effects on the production of proteins by Newcastle disease virus and encephalomyocarditis virus, demonstrating a functional role for RNF123 in regulating virus replication. Finally, in a series of carefully performed co-immunoprecipitation studies, the authors demonstrate that RNF123 blocks interaction between RIG-I/MDA5 and their signaling adaptor protein VISA. Overall, this paper identifies a novel interacting protein of RIG-I/MDA5, demonstrates its functional relevance in regulating viral replication in cells, and identifies a logical mechanism by which RNF123 controls RIG-I/MDA5 signaling. This paper is well-written, comprehensive, and describes what appears to be an important modulator of antiviral signaling. I have no major concerns regarding this manuscript and think that it is well-suited for publication in EMBO Reports.

Minor concern:
The authors may wish to foreshadow the involvement of VISA when describing the results presented in Figure 3. While the role of VISA is well-explained by later figures, Figure 3 was somewhat confusing when reading the paper in a linear fashion.
Referee #3:

Summary:

To shed light on the regulation of RIG-I and MDA5, the human sensors for viral RNA in the cytoplasm, the authors performed a yeast two-hybrid screen and received RNF123 as a hit. In their manuscript the authors present a plethora of data to confirm that RNF123 does indeed interact with and regulate RIG-I and MDA5 but not the cytosolic DNA sensor cGAS.

The authors show that overexpression of RNF123 in HEK293 cells does decrease the antiviral signaling of RIG-I and MDA agonists but not of overexpressed cGAS and STING using luciferase reporter assays for IFN-β and NF-κB signaling, Q-PCR, and a bioassay to quantify secreted IFN-β. The authors do also show that overexpression of RNF123 in HEK293 cells does increase the viral replication of viruses detected by RIG-I and MDA5 by fluorescence imaging and immunoblot.

Using the same methods the authors also show that knockdown of RNF123 in HEK293 and HeLa cells does increase the antiviral signaling of RIG-I and MDA agonists but not of cGAS and STING, and accordingly decreases viral replication of viruses detected by RIG-I and MDA5.

By overexpressing CARD domains of RIG-I and MDA5 the authors show that interaction of RNF123 does depend on these domains, whereas this interaction depends on the SPRY and the two coiled coil domains of RNF123. To confirm their conclusions, the authors also employ luciferase reporter assays for IFN-β and NF-κB signaling and co-immunoprecipitation experiments. Using the same methods, the authors additionally show that knockdown of RNF123 does not affect signaling due to TBK1, IRF3 or cGAS and STING overexpression. Furthermore the authors show with immunoblots that presence of RIG-I and MDA agonists increase the interaction of RIG-I and MDA5 with RNF123.

In the last part of their manuscript, the authors show via overexpression and co-immunoprecipitation experiments that RNF123 is inhibiting the interaction between RIG-I and MDA5 and the signaling adapter MAVS. The authors discuss the newly described mechanism of inhibitory regulation of RIG-I and MDA5 via RNF123 in the context of the list of existing RIG-I and MDA interaction partners and regulators and show that this mechanism is species specific.

The data presented by the authors of high quality and provides exhaustive proof for their claims. Therefore only minute adjustments are warranted.

Minor suggestions:

1. Please add the clone ID and catalog number of the antibodies used other scientists to verify the experiments.
2. Please correct inconsistency in the statistical analysis as a p value of 0.05 < p < 0.1 are indicated as non significant (n. s.) while at other times p < 0.1 is declared significant.
3. Please include a unit for their figures showing Q-PCR data, e.g. "fold-change".
4. Please also comment on the comparably stronger RIG-I band in the control IgG immunoprecipitations compared to the RNF123 immunoprecipitations (Fig.3E)

1st Revision - authors’ response 31 March 2016

Point by point responses:

Reviewer #1:

1. Western blots showing RNF123 protein levels should be included in Fig. 1.

Upon overexpression of RNF123 (100ng) a maximum 50% reduction in IFN induction is observed. Would a higher amount of RNF123 result in stronger inhibition of the signaling?

Reply:

We increased the amount of overexpressed RNF123 (200ng) and found a maximum 80% reduction in IFN-β or NF-κB induction. The RNF123 protein levels were shown under the charts in Fig 1A and 1B.
2. Figure 1F is not convincing. In addition, RNF123 expression levels should be also shown in the WB.

Reply:
We repeated experiments and re-presented Fig.1F. Overexpressed RNF123 could clearly enhanced the NDV-eGFP replication by GFP detection. And RNF123 expression levels were also shown in the WB.

3. SH-RNA-mediated knockdown efficiencies shown in Fig. 2 are very low. As a consequence, the phenotype observed is also very moderate. The authors should try to improve their KDs or to generate KO cell line by the CRISPR Cas9 technology. Reconstitution assays should also be performed to rule out off-target effects.

Reply:
Following the reviewer’s suggestion, we generated RNF123 knockout HEK293T cell line using the CRISPR/Cas9 system. We found that the phenotype observed in RNF123 knockout cells are much more pronounced than RNF123 knockdown cells. Activation of the IFN-β promoters induced by SeV and EMCV but not by cGAS and STING was strongly potentiated when RNF123 was deleted, whereas reconstitution of RNF123 expression plasmids could in turn dampened activation of IFN-β promoter. Consistent with this, RNF123 knockout also resulted in apparently improved transcription and secretion of endogenous IFN-β upon SeV and EMCV infection. We have shown these results in a new figure (Fig 3) in the revision.

4. Where is RNF123 localized within the cell? The authors should provide imaging data showing its localization with respect to Rig-I, MDA5 and MAVS during infection. This information would be a nice complement to the current version of the manuscript.

Reply:
Following the reviewer’s suggestion, we perform the immunofluorescent staining to identify the cellular localization of RNF123 with or without RIG-I, MDA5, and VISA. We showed that the overexpressed and endogenous RNF123 were localized dispersively in the cytoplasm of HeLa cells, which was similar to the distribution pattern of RIG-I and MDA5 but not VISA (Fig 4G, H). Double immunofluorescent staining showed that the overexpressed and endogenous RNF123 overlapped with RIG-I and MDA5, while there was no co-localization between RNF123 and VISA (Fig 4G, H). We also detected their localizations with SeV or EMCV infection and obtained the similar results (Fig EV2A, B). Nevertheless, we did not uncover obvious changes in the distributions upon SeV or EMCV infection. These data confirmed that RNF123 interacted with RIG-I and MDA5, but not VISA in the RLR signaling pathway.

5. How RNF123 inhibits RLR-MAVS binding is still unclear. Is RNF123 binding to the RLRs CARD domain inducible? The authors should better investigate what triggers this interaction to better clarify RNF123 mechanism of action.

Reply:
Following the reviewer’s suggestion, we perform the co-immunoprecipitation experiments to explore whether the interaction between RNF123 and RLRs CARD domain was enhanced by infection with SeV. The results showed that SeV infection improved association of overexpressed RNF123 with RIG-I CARDS (Fig 6C). As we did not found obvious changes in the distribution pattern of RNF123 upon SeV or EMCV infection (Fig 4G, H, EV2A, and EV2B) We speculated that there were conformational changes or modifications of the RNF123 upon virus infection, and these changes would improve the interaction between RNF123 and RIG-I/MDA5 CARDS. We Plan to use mass spectrography to further identify these speculations. We have added the above information to the discussion section (page 14).

Reviewer 2:
Minor concern:
The authors may wish to foreshadow the involvement of VISA when describing the results presented in Figure 3. While the role of VISA is well-explained by later figures, Figure 3 was somewhat confusing when reading the paper in a linear fashion.
Reply:
As the reviewer’s concern, we surely found that overexpression of RNF123 could partly inhibit the VISA-induced IFN-β and NF-κB promoters (Fig 4A, B). So we changed the description to “these results suggested that RNF123 may targets RIG-I and MDA5 in the RLR signaling pathway.” (page 8). Even so, we do not consider VISA is the target of RNF123, because knockdown of endogenous RNF123 specifically enhanced the activation of the IFN-β promoters induced by RIG-I/MDA5 but not VISA. Communoprecipitation experiments indicated that RIG-I and MDA5, but not VISA, associated with RNF123 (Fig 4D). Double immunofluorescent staining also showed that overexpressed or endogenous RNF123 had a similar distribution pattern and overlapped with RIG-I/MDA5, but there was no overlap between RNF123 and VISA (Fig 4G, H). All these data confirmed that RIG-I/MDA5 are the major regulatory targets of RNF123, although we could not completely exclude the possibility that RNF123 also acts on VISA. We have added the above points to the discussion section (page 13).

Reviewer 3:

1. Please add the clone ID and catalog number of the antibodies used other scientists to verify the experiments.
   Reply: We have added the shRNA clone ID and catalog number of antibodies in Materials and Methods section (page 15 and 19).

2. Please correct inconsistency in the statistical analysis as a p value of 0.05  < p  < 0.1 are indicated as non significant (n.s.) while at other times p < 0.1 is declared significant.
   Reply: We have corrected. All Asterisks indicate a significant difference level (Student’s t-test. ***, P <0.001; **, P <0.01; *, P <0.05; ns, P >0.05.)

3. Please include a unit for their figures showing Q-PCR data, e.g. "fold-change".
   Reply: We have changed to “IFN-β mRNA folds” for Q-PCR data.

4. Please also comment on the comparably stronger RIG-I band in the control IgG immunoprecipitations compared to the RNF123 immunoprecipitations (Fig.3E)
   Reply: For the reviewer’s concern, we have repeated this experiments and re-presented Fig 3E (Fig.4E in the revision). The band in the control IgG are much weaker than before. We considered it was a non-specific band, as its molecular weight is slightly larger than that of RIG-I. And the intensity of these band did not change with SeV infection (RIG-I was induced upon SeV infection).

2nd Editorial Decision 28 April 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the enclosed reports on it. As you will see, the referee now finds the manuscript suitable for publication in EMBO reports.

However, while crosschecking your manuscript I realized that some parts of the manuscript text are very similar to two of you older papers: doi: 10.1128/JVI.01073-13 and doi: 10.1074/jbc.M110.2066896. Some sentences where re-used without changes, some also from other publications. Please see the attached web-archive. We therefore ask you to go through your manuscript and re-write these parts using different wording, before we can proceed with the formal acceptance of your manuscript.
REFEE REPORTS

Referee #1:

The authors have adequately responded to the concerns of this reviewer, and the data as presented now are very convincing.

2nd Revision - authors' response 09 May 2016

Thank you for your advice. We have crosschecked our manuscript and tried our best to rewrite. We hope you find our revision acceptable for publication now.

3rd Editorial Decision 19 May 2016

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

Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

In the pink boxes below, please fill in the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

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 authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- A specification of the experimental system investigated (eg cell line, species name).
- The analysis(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 or unpaired), simple z-test, 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 tests, e.g., P-value = 0.05 and not P-value < 0.05 or 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.

B - Statistics and general methods

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

11. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

12. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

13. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

14. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment or pre-randomization procedures? If yes, please describe.

15. For animal studies, include a statement about randomization even if no randomization was used.

16. Were any steps taken to maximize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.

17. For animal studies, include a statement about blinding even if no blinding was done.

18. For every figure, are statistical tests justified as appropriate?

19. If the data meet the assumptions of the tests (e.g., normal distribution) Describe any methods used to assess this. We specified the tests used to calculate p-values in the inspection figure legend. (page 16, 25, 27, 29)

20. Is there an estimate of variation within each group of data?

21. If one variance statistic between the groups that are being statistically compared?

C - Reagents

...
**D- Animal Models**

6. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

7. Identify the committee(s) approving the study protocol.

8. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

9. We recommend the ARRIVE guidelines (PLoS Biol. 8(6), 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

10. We recommend consulting the M3C9-F guidelines (M3C9 Biol. Rev., 2005) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.

11. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

12. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

13. For experiments involving human subjects, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

14. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

15. Report the clinical trial registration number (e.g., ClinicalTrials.gov, or equivalent). Where applicable.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (available at CONSORT flow diagrams) and submit the CONSORT checklist (available at CONSORT checklist) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted these checks.

17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (Clin. Cancer Res. 15(22), 2009) for tumor marker studies. Please confirm you have followed these guidelines.

18. Provide accession codes for deposited data. See author guidelines, under ‘Data Deposition’.

19. 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
   f. Pharmacology and molecular interactions

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, each study should be deposited in one of the major public access-controlled repositories such as NCBI, EGA or EGA.

21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

22. Computational models that are central and integral to a study should be made available with no 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, each study should be deposited in one of the major public access-controlled repositories such as NCBI, EGA or EGA.

23. Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (APHIS/CDC) and list of select agents and toxins (APD/CD). According to our biosafety guidelines, provide a statement only if it could.