EMBO reports

RNF144A promotes antiviral responses by modulating STING ubiquitination

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Dear Dr. Wang,

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

As you will see, the referees state that these findings are of high interest. They have comments and suggestions to improve the study, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed, I will not detail them here. It will be critical, though, to carefully address point 4 of referee #3, i.e. to clarify the relationship between RNF144A, AMFR, and TRIM32 and why the same K236 residue of STING is targeted with different ubiquitin chains generated by different E3 ligases and what role this plays for STING trafficking.

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 detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We 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

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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:

In this study, the authors show that the E3 ubiquitin ligase RNF144A interact with STING and promoted its K6-linked ubiquitination at K236, thus promoting STING activation and anti-viral immune responses. Furthermore, the authors show that RNF144A and the STING K236 ubiquitination are pivotal for STING translocation and the subsequent STING-mediated antiviral responses. Intriguingly, the authors also reveal that RNF144A may be a potential target for treating aberrant type I IFN activity in SLE patients. The study uncovers a novel role of RNF144A in antiviral immunity by targeting STING and provides new ways for prevention and treatment of STING-related diseases (such as viral infections and SLE). Overall, the findings are novel and interesting. But the following concerns and questions should be addressed to further strengthen the study.

Specific points:

1. Figure 2, why the detection of infection in the lung, liver, and spleen, needs 24 h after HSV-1 infection, while in the brain it needs 4 days?
2. Figure3, RNF144A may have similar roles in regulating the immune responses triggered by poly (dA:dT), the authors should exclude the roles of AIM2 as well as cGAS and IFIT16 in this part.
3. Phosphorylation of STING plays vital roles in STING-induced DNA signaling pathway (doi:10.1038/s41586-019-1000-2; doi: 10.1126/science.aaa2630), the author should examine the roles of RNF144A in regulating STING phosphorylation.
4. Figure 7, the effects of RNF144A on the ubiquitination of endogenous in immune cells, such as BMDMs or peritoneal macrophages, should be examined.
5. Figure 7E, K29-linked ubiquitination of STING was also increased. The author should further explore the phenomenon or discussed it.
6. Figure8E & F, the "Dimer" blots seemed to be the phosphorylated STING.
7. In part of "Materials and Methods", the sequences of primers used in the study should be added.
8. In addition, the RNA or Gene writing of RNF144A, should be correct to "Rnf144a" , as well as others.
9.All the WB blots should be labelled with molecule weight.

Referee #2:

The manuscript by Yang et al entitled "RNF144A promotes cellular antiviral responses by manipulating the ubiquitination of STING" identifies the E3 ligase RNF144A as novel modulator of STING ubiquitination and antiviral defence upon HSV-1 infection. The authors start their analysis by demonstrating that RNF144A expression is upregulated upon DNA virus infection and that RNF144A is a regulator of IFN and ISG expression upon DNA virus infection. The authors created a RNF144A KO mouse model and investigated the role of RNF144A in controlling IFN and ISG responses in several cell types. The authors show that RNF144A interacts with STING, mediates it ubiquitination, localization and dimerization and finally demonstrate that RNF144A might act as a promotor of systemic lupus erythematous (SLE) in control and patient-derived SLE blood cells.

STING is a crucial regulator of interferon responses upon viral infection and a better understanding of how STING activity is controlled by ubiquitination is important and of interest for a wide audience. The manuscript of Yang et al aims to identify novel mechanisms of how RNF144A controls STING function, but several major issues remain unclear. Therefore, I think that this manuscript in its present form is not suitable for publication in EMBO Reports.

Points:
1. One of the major issues is that the manuscript remains highly descriptive and lacks mechanistic aspects of the RNF144A-STING interaction. The authors map the STING-RNF144A interaction and show that the interaction already occurs in the

absence of virus infection or DNA transfection. The added value of virus infection or DNA transfection remains unclear for ubiquitination.

2. In addition, most conclusions are based on overexpression experiments that might confound STING signalling. The authors should demonstrate that the observed effects of RNF144A on STING ubiquitination and function occur endogenous.

3. In Fig1, the authors describe that RNF114A expression is induced during virus infection. The authors should test if RNF144A is an ISG and if it is part of a feedforward loop to control the activity of STING, cytokine expression and antiviral defects. To validate the function of RNF144A and to really demonstrate that their findings are relevant and not the result of overexpression, the authors should reconstitute knockdown cells with WT and catalytically inactive RNF144A. Related to this, many RBR E3 ligases require a ubiquitin acceptor site for efficient E3 ligase function and the authors should reconstitute this mutation in their experiments. In addition, the use of siRNA does not give really clear answers and the authors should use CRISPR to generate full and stable RNF144A knockout.

4. In many cases, it remains unclear if RNF144A affects viral uptake, replication or the formation of novel viral particles. In many experiments, the authors do not use PCR to monitor HSV-1 DNA. The authors should carefully work these processes out in a systematic manner.

5. In Fig2, the authors generate a RNF144A KO mouse model, but do not discuss any phenotype related to STING function. It would be interesting to see what the effects are of loss of STING in RNF144A KO cells. In F2E it remains unclear which organ was tested. Here, it also remains unclear if RNF144A affects viral uptake, replication or the formation of novel viral particles. The authors should carefully work these processes out in a systematic manner.

6. In Fig4, the authors should reconstitute their KO MEFs with WT RNF144A and the above-mentioned RNF144A mutants to really control their observations. The panel for F4J is missing.

7. Panel F5E is not clear and does not show any difference between WT and KO.

8. The authors describe an interaction between RNF144A and STING, but it remains unclear if this occurs endogenous. The authors should demonstrate more clearly that this interaction occurs without overexpression. In addition, the authors should demonstrate where in the cells this interaction occurs.

9. The ubiquitination experiments are all done with overexpressed plasmids. The authors should show that STING ubiquitination at K236 by RNF114A happens endogenous by mass-spectrometric approaches.

10. The SLE experiments are interesting but stand alone with respect to the other findings. Connecting these findings with STING ubiquitination would be interesting and justify more why the authors especially looked into SLE.

11. It remains unclear if STING ubiquitination occurs directly by RNF144A or if it a consequence of more STING induced by RNF144A-mediated increases of IFNs during virus infection. The authors should address these effects by blocking auto- and paracrine IFN signalling, and analyse RNF144A-mediate STING ubiquitination.

12. The number of figures is too much and should be combined/condensed/restructured.

Referee #3:

In this manuscript, the authors reported that RNF144A, an E3 ligase, K6-ubiquitinated STING and that this ubiquitination was essential for STING translocation from the ER to the Golgi. The results are potentially interesting, but several major issues need to be clarified.

Major critiques:
(1) This reviewer was concerned about the site of action of RNF144A. The authors suggested that the interference of RNF144A with cGAS was negligible (Fig. 6B and Fig. EV4A/B). The authors should also quantitate the amount of cGAMP in dsDNA-stimulated cells.

(2) All the imaging data should be quantitated with the Pearson co-efficient. It is extremely hard to interpret the data without quantitation. For example, In Fig. 8C, some of KO cells with infection of HSV-1, showed the good colocalization of STING with GM130.

(3) The key WB data should be quantitated. For example, the difference of the intensity of pIRF3 between SC- or A2-treated cells (Fig. EV4A/B) appeared very subtle.

(4) Regarding the regulation of STING trafficking out of the ER by ubiquitination, several reports have been published, as the authors discussed in Discussion. The apparent problem is that the same K236 residue with different Ub chain generated by different E3 ligases is suggested to play a role in the STING trafficking out of the ER. The authors should investigate and clarify the relationship between RNF144A, AMFR, and TRIM32.

(5) The K6-ubiquination of STING by RNF144A occurred even in the presence of BFA?

Minor critiques:
(1) Introduction needs to address the precedent publications about STING ubiquitination and its involvement of STING trafficking, signalling, and degradation.

(2) Konno et al. indicated that K224 ubiquitination by MUL1 was essential for STING trafficking (Sci Immunol 2017). At least, the report was cited and discussed.

(3) Some the data in main Figures can be transferred to suppelmentary Figures. For example, Figure 3 and 4 can be merged.

Editor：

As you will see, the referees state that these findings are of high interest. They have comments and suggestions to improve the study, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed, I will not detail them here. It will be critical, though, to carefully address point 4 of referee #3, i.e. to clarify the relationship between RNF144A, AMFR, and TRIM32 and why the same K236 residue of STING is targeted with different ubiquitin chains generated by different E3 ligases and what role this plays for STING trafficking.

Response: Thank you for the comments. This is a very good question. It would be very interesting if the relationship between RNF144A, AMFR, and TRIM32 is clarified. Thus, we did various experiments to address this point. Firstly, we explored whether RNF144A interacted with AMFR or Trim32. As shown in Figure N1A, exogenously expressed RNF144A could co-immunoprecipitated with AMFR, but not Trim32, suggesting RNF144A might interact with AMFR. However, in HSV-1 infected THP1 cells, no endogenous interaction between RNF144A and AMFR or Trim32 was detected (Figure N1B), suggesting the exogenous interaction between RNF144A and AMFR might be artificial. Next, we examined whether RNF144A competed with AMFR or Trim32 for the interaction with STING. As shown in Figure N1C, we did not find RNF144A had a significant effect on the interaction between STING and AMFR or Trim32. Then, we investigated the effect of RNF144A on the function of AMFR and Trim32. HEK293T cells were transfected with cGAS and STING, together with indicated plasmids for 24 h, and IFN-β expression was evaluated by real-time PCR. As expected, RNF144A, AMFR, and Trim32 promoted cGAS-STING-induced IFN-β expression separately. However, RNF144A only promoted AMFR- or Trim32-induced IFN-β expression slightly, even not as strongly as RNF144A did by itself (Figure N1D). Then we explored whether the function of AMFR or Trim32 was dependent on RNF144A. Thus, we knocked down AMFR or Trim32 expression in *Rnf144a*-deficient MEFs and explored the effect of RNF144A deficiency on the function of AMFR or

Trm32. The knockdown efficiency is shown in Figure N1E. Here, we noticed that, when AMFR or Trim32 was knocked down, RNF144A still had a strong effect on HSV-1-induced anti-viral immune responses. However, in *Rnf144a*-deficient MEFs, AMFR or Trim32 knockdown only inhibited HSV-1-induced anti-viral immune responses very slightly. It seems that RNF144A might be required for the full function of AMFR and Trim32, but further study is required to draw a conclusion.

Additionally, Wang et al. reported that using the two-step immunoprecipitation assay, they failed to detect any ubiquitination signal of STING in the presence of Trim32 and they considered that Trim32 did not catalyze the polyubiquitination of STING per se but might promote the poly-ubiquitination of other proteins in the STING complex (Immunity 41, 919–933, December 18, 2014). In the same article, Wang et al. identified that other than K236, AMFR also promoted the ubiquitination of STING at K137, K150, and K224 (Immunity 41, 919–933, December 18, 2014). These results may suggest that Trim32 and AMFR do not have to compete with RNF144A for the modification of STING at K236 to exert their roles in signal transduction.

Taken together, till now, we did not find evidence strong enough to draw a conclusion about the relationship between RNF144A, AMFR, and Trim32. I think it may be better to address this question by using double- or triple-knockout mice. Considering we do not have such mice now and it will need a long period to generate these mice, we did not do these experiments, and we did not add these preliminary data to the manuscript. Instead, we revised the discussion about the K236, to avoid the misunderstanding that the K236 lysine residue of STING is the only target of Trim32 and AMFR.

The description of K236 was rewritten as “It has been reported that another two E3 ubiquitin ligases, autocrine motility factor receptor (AMFR) and TRIM32, also targeted STING for ubiquitination at lysine residues including K236. AMFR promoted the K27-linked polyubiquitination of STING, whereas TRIM32 catalyzed the K63-linked polyubiquitination of STING (Wang et al, 2014; Zhang et al, 2012). However, Wang et al. reported that using the two-step immunoprecipitation assay,

they failed to detect any ubiquitination signal of STING in the presence of Trim32 and they considered that Trim32 did not catalyze the polyubiquitination of STING per se but might promote the poly-ubiquitination of other proteins in the STING complex (Immunity 41, 919–933, December 18, 2014). Additionally, other than K236, AMFR also promoted the ubiquitination of STING at K137, K150, and K224 (Immunity 41, 919–933, December 18, 2014). These results may suggest that the modifications at K236 of STING are not exactly the same and redundant, but it remains unclear how the host cells organize these modifications on STING."

Figure for referee with unpublished data and its description has been removed upon request by the authors.

Referee #1:

In this study, the authors show that the E3 ubiquitin ligase RNF144A interact with STING and promoted its K6-linked ubiquitination at K236, thus promoting STING activation and anti-viral immune responses. Furthermore, the authors show that RNF144A and the STING K236 ubiquitination are pivotal for STING translocation and the subsequent STING-mediated antiviral responses. Intriguingly, the authors also reveal that RNF144A may be a potential target for treating aberrant type I IFN activity in SLE patients. The study uncovers a novel role of RNF144A in antiviral immunity by targeting STING and provides new ways for prevention and treatment of STING-related diseases (such as viral infections and SLE). Overall, the findings are novel and interesting. But the following concerns and questions should be addressed to further strengthen the study.

Specific points:

1. Figure 2, why the detection of infection in the lung, liver, and spleen, needs 24 h after HSV-1 infection, while in the brain it needs 4 days?

Response: This question is very good. We chose these time points according to the report by Zhang et al. (Cell Research (2020) 0:1–14; https://doi.org/10.1038/s41422-020-0341-6), in which they detected IFN-β expression in lungs 24h after infection and in brain 4 days after infection. In some other reports, in response to HSV-1 infection, IFN-β expression in the lung, liver, and spleen was usually detected from 12h to 2 days after infection and in the brain, it was usually detected around 3 days after infection (Nat Communications. 2020 Jul 7;11(1):3382., Nat Commun . 2020 Nov 2;11(1):5536. doi: 10.1038/s41467-020-19318-3., PLoS Pathog. 2021 Mar 15;17(3):e1009401. doi: 10.1371/journal.ppat.1009401., PLoS Pathog. 2020 Jan 22;16(1):e1008178. doi: 10.1371/journal.ppat.1008178., Sci Adv. 2022 Jan 28;8(4):eabh0496. doi: 10.1126/sciadv.abh0496.). I think the existence of the blood-brain barrier might be the reason why usually it takes a longer period to detect anti-viral responses in the brain than in the lung, liver, and spleen.

2. Figure3, RNF144A may have similar roles in regulating the immune responses triggered by poly (dA:dT), the authors should exclude the roles of AIM2 as well as cGAS and IFIT16 in this part.

Response: This is a very good question. AIM2 has been identified by several groups as a cytosolic receptor for double-stranded DNA (dsDNA). However, as far as we know, usually, AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC, leading to the secretion of IL-1β and IL-18 (Fernandes-Alnemri T, et al. Nature 2009., Nature 2009 Mar 26;458(7237):514-8. doi: 10.1038/nature07725, Exp Dermatol . 2011 Dec;20(12):1027-9. doi: 10.1111/j.1600-0625.2011.01382.x.), which is not the main point of our story. In our

manuscript, we focused on the effect of RNF144A on the secretion of type I IFN, TNF-α, and IL-6. Further, we exhibited that in *STING*-deficient THP1 cells, RNF144A made no significant difference in the HSV-1-induced innate immune responses. Thus, we did not exclude the roles of AIM2 or other NLRs in this part.

3. Phosphorylation of STING plays vital roles in STING-induced DNA signaling pathway (doi:10.1038/s41586-019-1000-2; doi: 10.1126/science.aaa2630), the author should examine the roles of RNF144A in regulating STING phosphorylation.

Response: The reviewer`s suggestion is very good and has been well taken. We added the data of STING phosphorylation to Figures 1J, 3G, 3H, 4H, 4I, EV1G, EV2E, and EV2F. In all these Figures, the patterns of STING phosphorylation were similar to the patterns of IRF3 or TBK1 phosphorylation.

Figure 1J

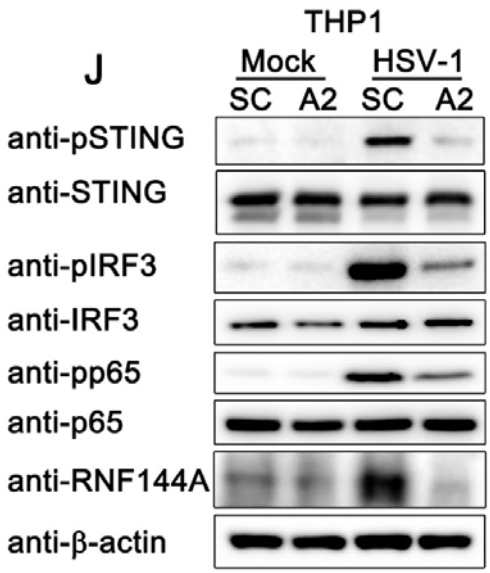


Figure 1 RNF144A promotes DNA virus- or exogenous cytosolic DNA-triggered innate immune responses.

(J) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with HSV-1 for 8 h. The cells were lysed for

immunoblot assays. β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

Figure 3G and Figure 3H

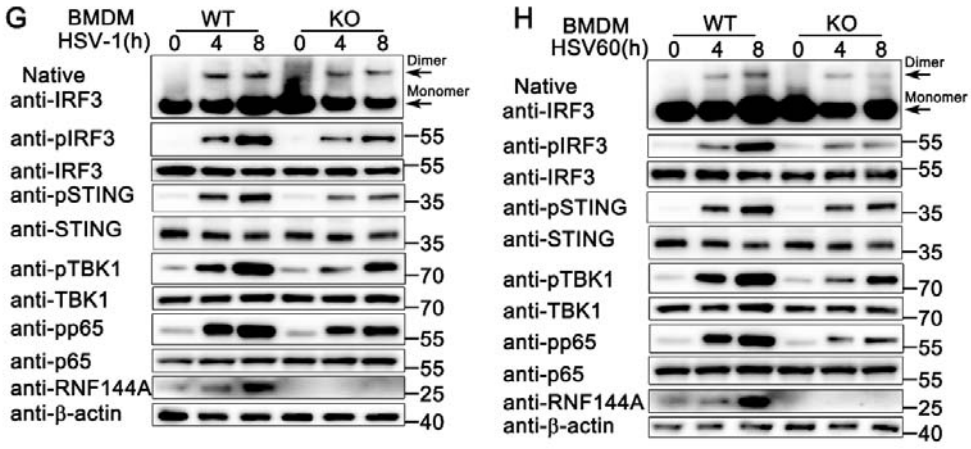


Figure 3 RNF144A deficiency impairs DNA virus or exogenous cytosolic DNA-triggered innate immune responses in BMDMs.

(G) Wild-type (WT) and *Rnf144a*-deficient (KO) BMDMs were infected with HSV-1 (MOI=1) for indicated periods. Then Native-PAGE and SDS-PAGE assays were performed.

(H) Wild-type (WT) and *Rnf144a*-deficient (KO) BMDMs were transfected with HSV60 (1μg/ml) for indicated periods. Then Native-PAGE and SDS-PAGE assays were performed.

β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

Figure 4H and Figure 4I

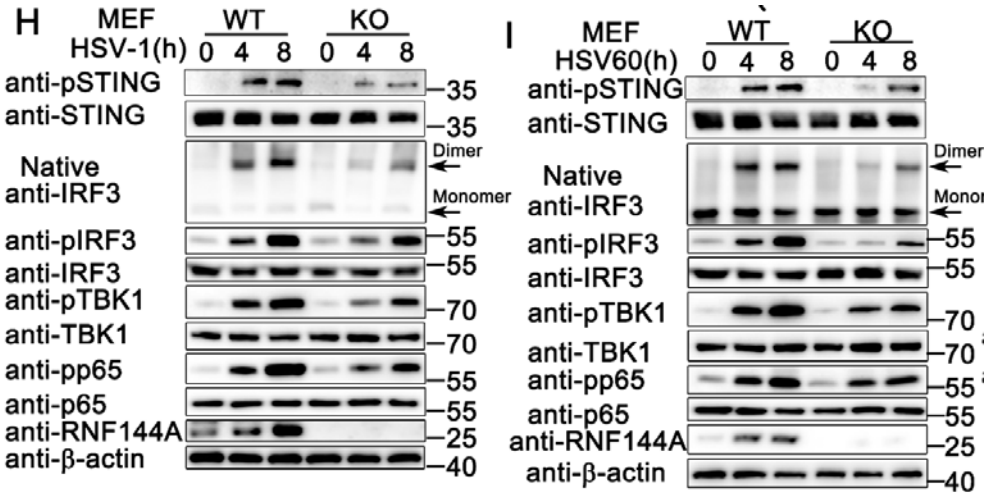


Figure 4 RNF144A deficiency impairs DNA virus- or exogenous cytosolic DNA-triggered innate immune responses in MEFs.

(H) Wild-type (WT) and *Rnf144a*-deficient (KO) MEFs were infected with HSV-1 (MOI=1) for indicated periods. Then Native-PAGE and SDS-PAGE assays were performed.

(I) Wild-type (WT) and *Rnf144a*-deficient (KO) MEFs were transfected with HSV60 (1μg/ml) for indicated periods. Then Native-PAGE and SDS-PAGE assays were performed.

β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

Figures EV1G

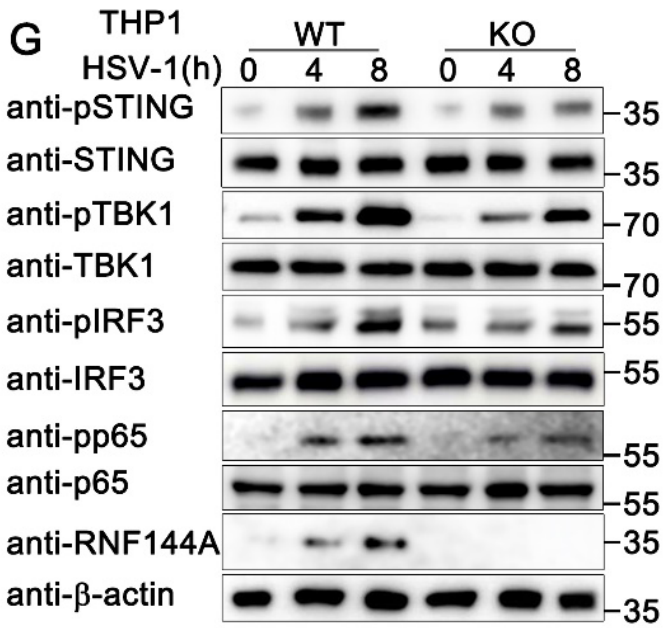


Figure EV1 RNF144A promotes DNA virus- or exogenous cytosolic DNA-triggered innate immune responses.

(G) Wild-type (WT) and *RNF144A*-deficient (KO) PMA-THP1 cells were infected with HSV-1 (MOI=1) for indicated periods. Then the immunoblot assays were performed. β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

Figure EV2E and Figure EV2F

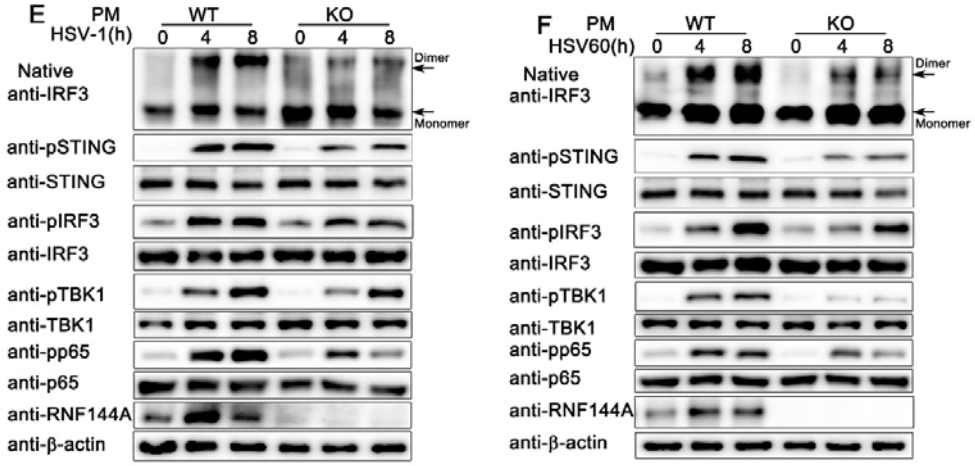


Figure EV2 RNF144A deficiency impairs DNA virus or exogenous cytosolic DNA-triggered innate immune responses in PMs.

(E, F) Wild-type (WT) and *Rnf144a*-deficient (KO) PMs were infected with HSV-1

(MOI=1) (E) or transfected with HSV60 (1μg/ml) (F) for indicated periods. Then Native-PAGE and SDS-PAGE assays were performed.

β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

4. Figure 7, the effects of RNF144A on the ubiquitination of endogenous in immune cells, such as BMDMs or peritoneal macrophages, should be examined.

Response: The reviewer`s suggestion is very good and has been well taken. We examined the effects of RNF144A on the ubiquitination of endogenous STING in PMA-THP1 cells, BMDMs, and peritoneal macrophages. Further, RNF144A knockdown in PMA-THP1 cells decreased HSV-1-induced ubiquitination of STING (Figure 6D). Next, we examined the effects of RNF144A on the ubiquitination of endogenous STING in MEFs, BMDMs, and PMs. Compared to control wild-type cells, *Rnf144a*-deficient MEFs, BMDMs, or PMs displayed impairment in HSV-1-induced ubiquitination of STING (Figures 6E, EV5D and EV5E).

Figure 6D

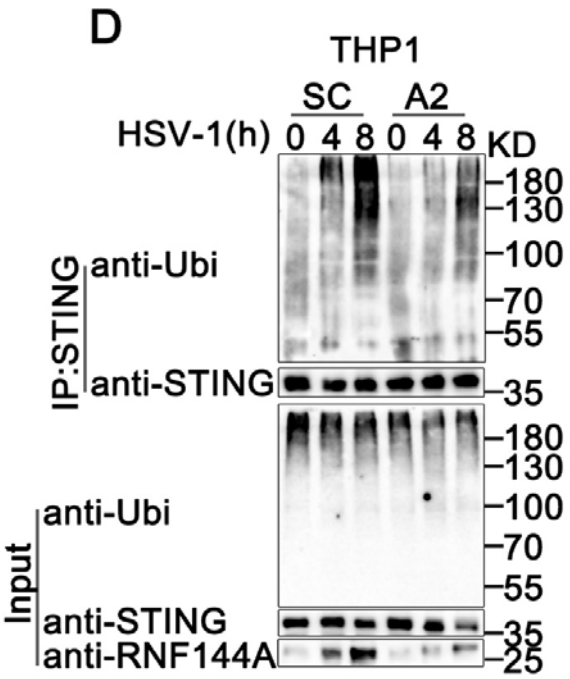


Figure 6 RNF144A promotes the ubiquitination of STING

(D) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2). At 24 h after transfection, the cells were infected with HSV-1 (MOI=1) for indicated periods. Afterward, immunoprecipitation (IP) and immunoblot (IB) assays were performed.

The data are representative of three independent experiments.

Figure EV5D and Figure EV5E

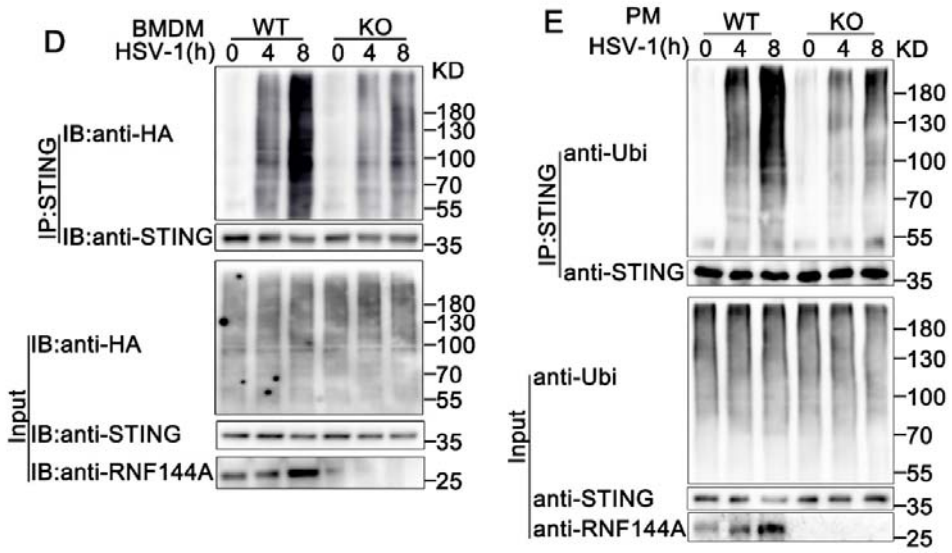


Figure EV5 RNF144A promotes the ubiquitination of STING.

(D, E) Wild-type (WT) and *Rnf144a*-deficient (KO) BMDMs (D) or PMs (E) were infected with HSV-1 (MOI=1) for indicated periods. Afterward, the cells were lysed and subjected to immunoprecipitation (IP) and immunoblot (IB) assays.

The data are representative of three independent experiments.

5. Figure 7E, K29-linked ubiquitination of STING was also increased. The author should further explore the phenomenon or discussed it.

Response: The reviewer`s suggestion is very good and has been well taken. We used the K29R mutant of ubiquitin to explore the phenomenon. This phenomenon was further confirmed by the usage of the K6R (only the Lys residue 6 was mutated to Arg), K29R (only the Lys residue 29 was mutated to Arg), and K48R (only the Lys residue 48 was mutated to Arg) mutants of ubiquitin. Immunoprecipitation and immunoblot analysis indicated that RNF144A increased K48R or K29R-mediated ubiquitination of STING, but not K6R, indicating the Lys residue 6 was essential to the RNF144A-triggered linkage of STING with ubiquitin (Fig 6H).

Figure 6H

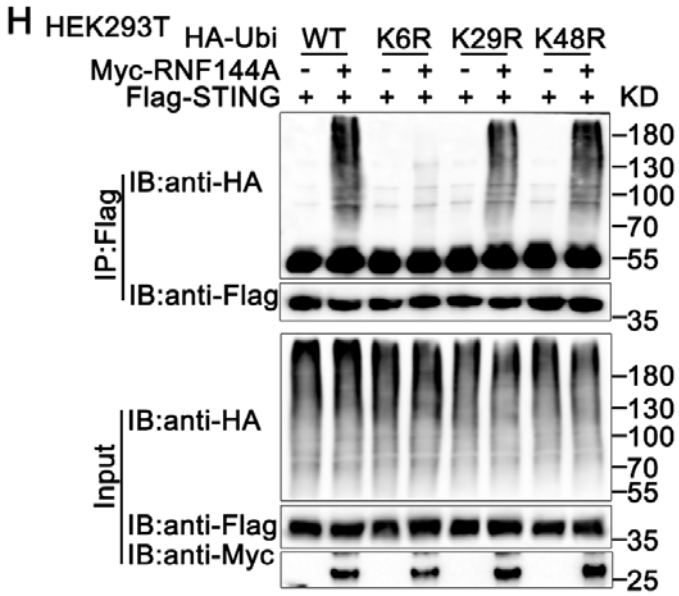


Figure 6 RNF144A promotes the ubiquitination of STING

(H) HEK293T cells were transfected with various combinations of plasmids as indicated. 24 h later, immunoprecipitation (IP) and immunoblot (IB) assays were performed.

The data are representative of three independent experiments.

6. Figure8E & F, the "Dimer" blots seemed to be the phosphorylated STING.

Response: The reviewer`s point is very good and we have double-checked these blots. The blots of phosphorylated STING, as shown in Figure N2, detected by the anti-pSTING, were about 40KD, whereas the blots of STING dimer, as shown in Figure N3, detected by the anti-STING, were about 80KD.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

7. In part of "Materials and Methods", the sequences of primers used in the study should be added.

Response: The reviewer`s suggestion is very good and has been well taken.

We added Appendix Table S1 including the sequences of primers used in the study.

Appendix Table S1 Primers for real-time PCR assays

Gene name	Primer sequence (5'→3') Forward（SP）	Primer sequence (5'→3') Reverse（AS）
Human *CCL5*	TACACCAGTGGCAAGTGCTC	ACACACTTGGCGGTTCTTTC
Human *CXCL10*	GGTGAGAAGAGATGTCTGAATCC	GTCCATCCTTGGAAGCACTGCA
Human *GAPDH*	TCAACGACCACTTTGTCAAGCTCA	GCTGGTGGTCCAGGTCTTACT
Human *IFIT1*	GCCATTTTCTTTGCTTCCCCTA	TGCCCTTTTGTAGCCTCCTTG
Human *IFNB*	CACGACAGCTCTTTCCATGA	AGCCAGTGCTCGATGAATCT
Human *RNF144A*	GAGCAGATGACAACCATAGCC	TGCACTCAATCTCGTTCTCCT
Human *TNF*	GGCGTGGAGCTGAGAGATAAC	GGTGTGGGTGAGGAGCACAT
Mouse *Ccl5*	TCACCATATGGCTCGGACACCAC	TTGGCACACACTTGGCGGTTC
Mouse *Cxcl10*	ATCATCCCTGCGAGCCTATCCT	GACCTTTTTTGGCTAAACGCTTTC
Mouse *Gapdh*	ACGGCCGCATCTTCTTGTGCA	ACGGCCAAATCCGTTCACACC
Mouse *Ifit1*	ACAGCAACCATGGGAGAGAATGCTG	ACGTAGGCCAGGAGGTTGTGCAT
Mouse *Ifnb*	TCCTGCTGTGCTTCTCCACCACA	AAGTCCGCCCTGTAGGTGAGGTT
Mouse *Il6*	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
HSV-1 gDNA	TGGGACACATGCCTTCTTGG	ACCCTTAGTCAGACTCTGTTACTTACCC
HSV-1 *UL30*	AGAGGGACATCCAGGACTTTGT	CAGGCGCTTGTTGGTGTAC

8. In addition, the RNA or Gene writing of RNF144A, should be correct to "Rnf144a" , as well as others.

Response: The reviewer`s suggestion is very good and has been well taken. We have

checked and revised all the RNA or Gene writing throughout the manuscript.

9.All the WB blots should be labelled with molecule weight.

Response: The reviewer`s suggestion is very good and has been well taken. We have labeled the blots with molecule weight throughout the manuscript.

Referee #2:

The manuscript by Yang et al entitled "RNF144A promotes cellular antiviral responses by manipulating the ubiquitination of STING" identifies the E3 ligase RNF144A as novel modulator of STING ubiquitination and antiviral defence upon HSV-1 infection. The authors start their analysis by demonstrating that RNF144A expression is upregulated upon DNA virus infection and that RNF144A is a regulator of IFN and ISG expression upon DNA virus infection. The authors created a RNF144A KO mouse model and investigated the role of RNF144A in controlling IFN and ISG responses in several cell types. The authors show that RNF144A interacts with STING, mediates it ubiquitination, localization and dimerization and finally demonstrate that RNF144A might act as a promotor of systemic lupus erythematous (SLE) in control and patient-derived SLE blood cells.

STING is a crucial regulator of interferon responses upon viral infection and a better understanding of how STING activity is controlled by ubiquitination is important and of interest for a wide audience. The manuscript of Yang et al aims to identify novel mechanisms of how RNF144A controls STING function, but several major issues remain unclear. Therefore, I think that this manuscript in its present form is not suitable for publication in EMBO Reports.

Points:

1. One of the major issues is that the manuscript remains highly descriptive and lacks

mechanistic aspects of the RNF144A-STING interaction. The authors map the STING-RNF144A interaction and show that the interaction already occurs in the absence of virus infection or DNA transfection. The added value of virus infection or DNA transfection remains unclear for ubiquitination.

Response: This is a very good question. As shown in Figure N4, with the treatment of Fludarabine, the inhibitor of STAT1 activation, RNF144A and STING could not be induced by HSV-1 infection. Then, we examined the ubiquitination of STING in the presence of Fludarabine and the results indicated that HSV-1 infection increased the ubiquitination of STING and RNF144A deficiency decreased the ubiquitination of STING when the expression of STING exhibited no significant difference between wild-type and *Rnf144a*-deficient cells (Figure N5). However, in the study of our lab and other groups, HSV-1 infection or viral DNA transfection did not increase STING expression, but decreased STING expression (PLoS Pathog. 2020 Mar 3, PLoS Pathog. 2015 Jun 26, Cell Discov. 2018 Mar 20). Further, our data did not suggest RNF144A affected STING expression, for example, in Figures 1J, 3G-H, 6D, 7A-B, 7D-H, and 8G-H. Thus, we did not put the Fludarabine data into our manuscript.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

2. In addition, most conclusions are based on overexpression experiments that might confound STING signalling. The authors should demonstrate that the observed effects of RNF144A on STING ubiquitination and function occur endogenous.

Response: The reviewer`s suggestion is very good and has been well taken. In the previous version, we showed the effects of RNF144A on STING ubiquitination in Figure 7D. In the revised version, we added the new Figures 6D, EV5D, and EV5E to further address this point. As shown in Figure 6D, RNF144A knockdown decreased HSV-1-induced ubiquitination of STING. Next, we examined the effects of RNF144A on the ubiquitination of endogenous STING in MEFs, BMDMs, and PMs. Compared to

control wild-type cells, *Rnf144a*-deficient MEFs, BMDMs, or PMs displayed impairment in HSV-1-induced ubiquitination of STING (Figures 7E, EV5D and EV5E).

Figure 6D

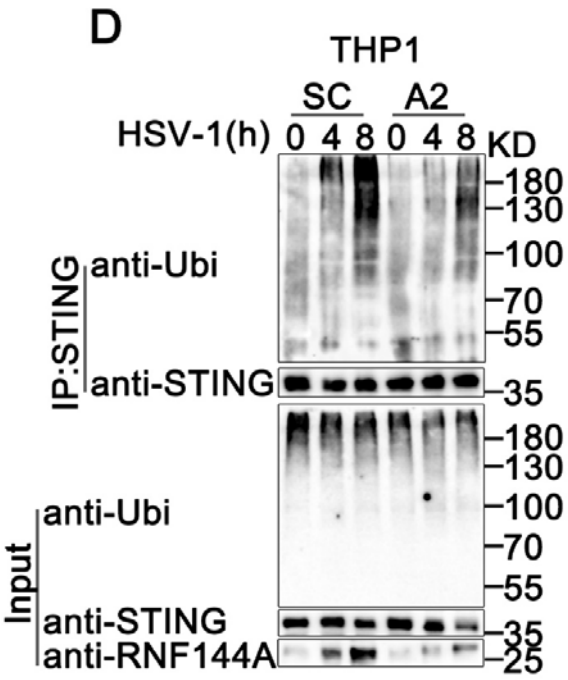


Figure 6 RNF144A promotes the ubiquitination of STING

(D) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2). At 24 h after transfection, the cells were infected with HSV-1 (MOI=1) for indicated periods. Afterward, immunoprecipitation (IP) and immunoblot (IB) assays were performed.

The data are representative of three independent experiments.

Figure EV5D and Figure EV5E

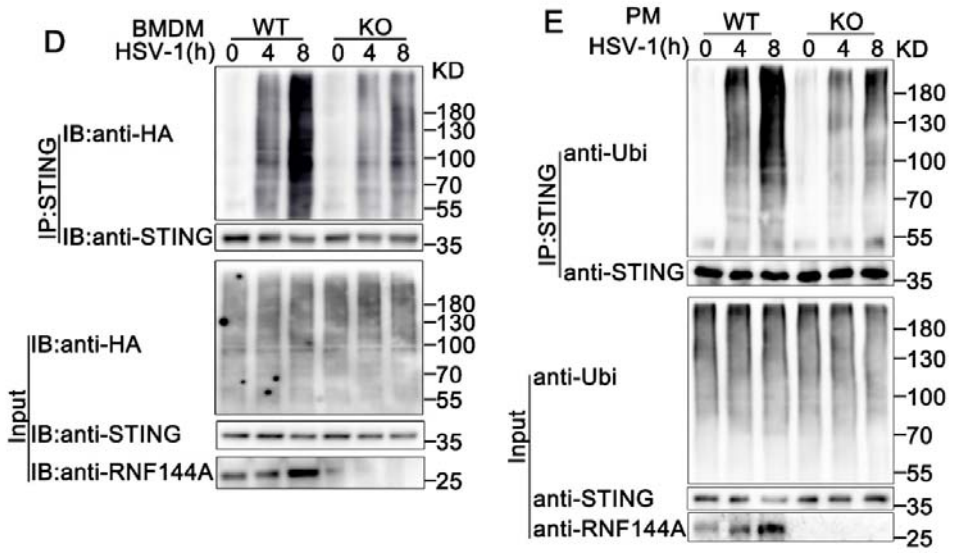


Figure EV5 RNF144A promotes the ubiquitination of STING.

(D, E) Wild-type (WT) and *Rnf144a*-deficient (KO) BMDMs (D) or PMs (E) were infected with HSV-1 (MOI=1) for indicated periods. Afterward, the cells were lysed and subjected to immunoprecipitation (IP) and immunoblot (IB) assays.

The data are representative of three independent experiments.

3. In Fig1, the authors describe that RNF114A expression is induced during virus infection. The authors should test if RNF144A is an ISG and if it is part of a feedforward loop to control the activity of STING, cytokine expression and antiviral defects.

Response: This is a very good question. We stimulated PMA-THP1 cells with IFN-β and the expression of RNF144A was examined. As shown in Figure N6, RNF144A was induced by IFN-β stimulation, suggesting RNF144A might be an ISG and part of a feed-forward loop to control the activity of STING, cytokine expression, and antiviral defense.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

To validate the function of RNF144A and to demonstrate that their findings are relevant and not the result of overexpression, the authors should reconstitute knockdown cells with WT and catalytically inactive RNF144A. Related to this, many RBR E3 ligases require a ubiquitin acceptor site for efficient E3 ligase function and the authors should reconstitute this mutation in their experiments.

Response: This is a very good question. In the previous version, we already reconstituted the KO MEFs with WT RNF144A and the above-mentioned RNF144A mutants in Figures 7B and 7C (Figures 6B and 6C in the revised version). Wild-type RNF144A and its mutants were transfected into *Rnf144a*-deficient MEFs and the effect of these ligase-dead mutants was examined. As expected, the C20/23A and C198A mutants of RNF144A lost the ability to increase the ubiquitination of endogenous STING in MEFs (Fig 6B). Further, neither C20/23A nor C198A mutant of RNF144A increased HSV60-induced production of IFN-β and IP-10 as wild-type RNF144A did, indicating the integrity of RING domain was required for the role of RNF144A in STING ubiquitination and STING-mediated signaling pathway (Fig 6C).

Figures 6B and 6C

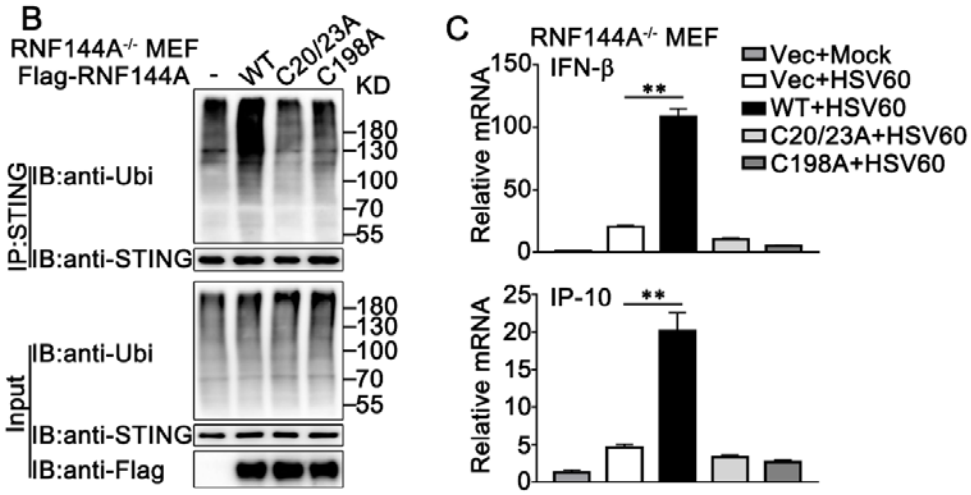


Figure 6 RNF144A promotes the ubiquitination of STING

(B) *Rnf144a*-deficient (KO) MEFs were transfected with various combinations of plasmids as indicated. 24 h later, immunoprecipitation (IP) and immunoblot (IB) assays were performed.

(C) *Rnf144a*-deficient (KO) MEFs were transfected with empty vector (Vec), wild-type RNF144A plasmid, or its mutants for 24 h and then transfected with HSV60 (1□g/ml) for 8 h. Then the cells were lysed for real-time PCR assays.

The data are representative of three independent experiments and are presented as mean ± SD. **, $p < 0.01$.

In addition, the use of siRNA does not give really clear answers and the authors should use CRISPR to generate full and stable RNF144A knockout.

Response: This is a very good suggestion and has been well taken. *RNF144A*-KO THP1 cells were generated by CRISPR and Figures EV1D-G were added to address this point. The expression of RNF144A could not be detected in *RNF144A*-deficient PMA-THP1 cells as suggested by immunoblot assays (Figure EV1D). Compared to wild-type PMA-THP1 cells, upon HSV-1 infection, *RNF144A*-deficient cells exhibited decreased anti-viral innate immune responses (Figures EV1E-G).

Figures EV1D-G

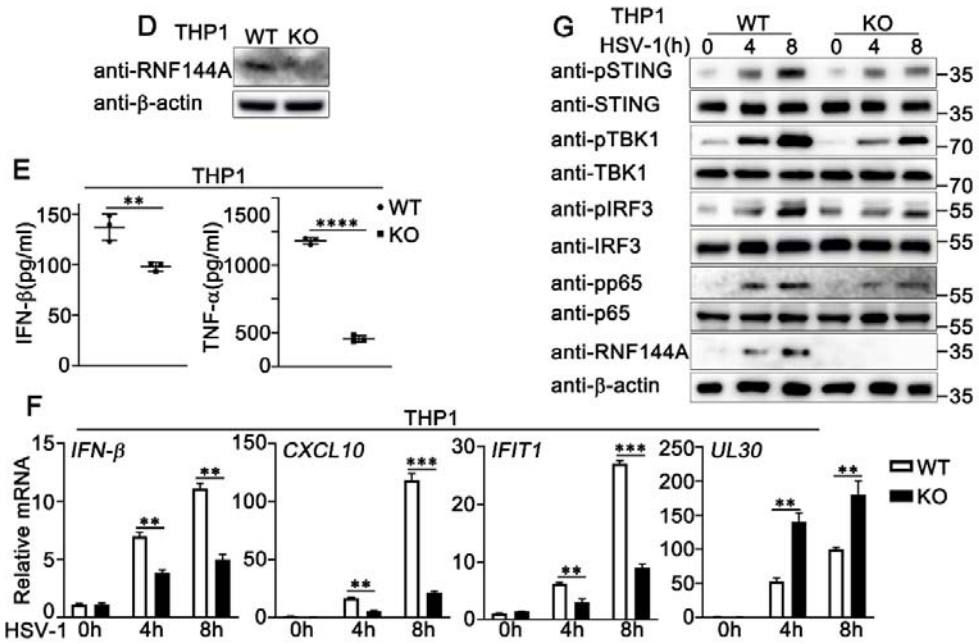


Figure EV1 RNF144A promotes DNA virus- or exogenous cytosolic DNA-triggered innate immune responses.

(D) Wild-type (WT) and *RNF144A*-deficient (KO) PMA-THP1 cells were infected with HSV-1 (MOI=1) for 8 h. The cells were lysed for immunoblot assays.

(E) Wild-type (WT) and *RNF144A*-deficient (KO) PMA-THP1 cells were infected with HSV-1 (MOI=1) for 24 h. Then the supernatants were collected and subjected to ELISA assays.

(F) Wild-type (WT) and *RNF144A*-deficient (KO) PMA-THP1 cells were infected with HSV-1 (MOI=1) for indicated periods. Then the cells were lysed for real-time PCR assays.

(G) Wild-type (WT) and *RNF144A*-deficient (KO) PMA-THP1 cells were infected with HSV-1 (MOI=1) for indicated periods. Then the immunoblot assays were performed. β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments and are presented as mean ± SD. **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$.

4. In many cases, it remains unclear if RNF144A affects viral uptake, replication or the

formation of novel viral particles. In many experiments, the authors do not use PCR to monitor HSV-1 DNA. The authors should carefully work these processes out in a systematic manner.

Response: This is a very good suggestion and has been well taken. We added Figures EV4K, EV4L, and EV4M to address this point. Additionally, in *STING*-deficient THP1 cells, RNF144A knockdown did not affect HSV-1-induced production of IFN-β, IP-10, ISG56, and IL-6 (Fig EV4K), and HSV-1 infection (Fig EV4K-M), indicating that the effect of RNF144A on HSV-1 infection was dependent on the existence of STING and innate immune responses, not by affecting virus directly. Additionally, we added HSV-1 gDNA and HSV-1 UL30 results to the manuscript, such as Figures 1H, 3B, and 4B.

Figures EV4K-M

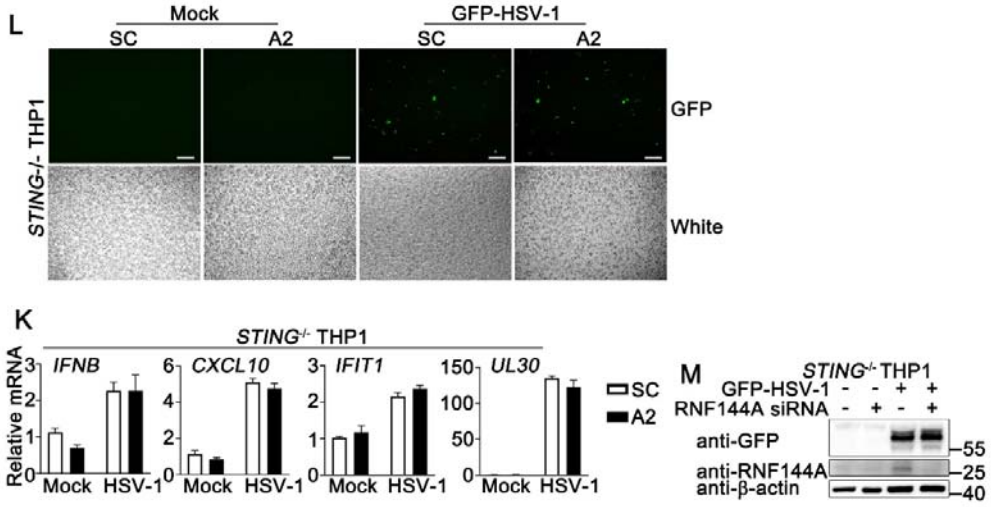


Figure EV4 RNF144A regulates STING-mediated signaling pathways.

(K) *STING*-deficient (KO) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with HSV-1 for 8 h. Afterward, the cells were lysed for real-time PCR assays.

(L, M) *STING*-deficient (KO) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with GFP-HSV-1 for 24 h.

Afterward, the cells were subjected to image taken (L) or immunoblot assays (M). β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments and are presented as mean ± SD.

Figure 1H

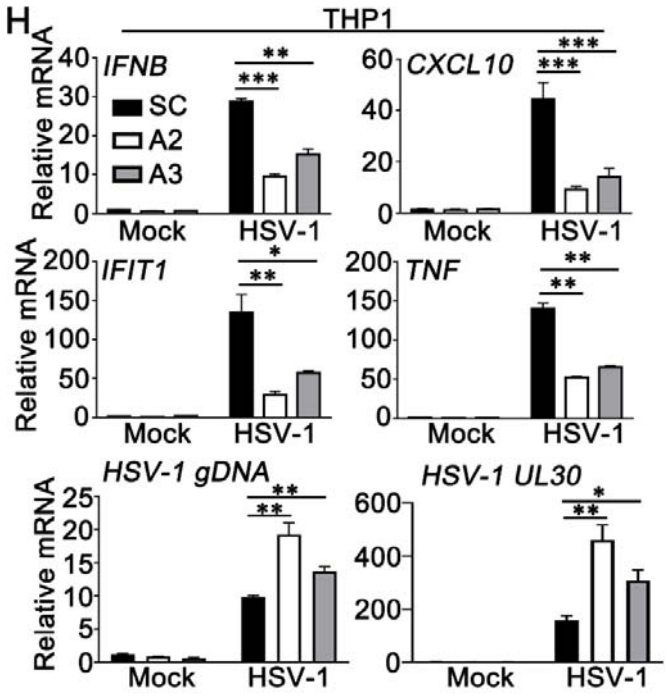


Figure 1 RNF144A promotes DNA virus- or exogenous cytosolic DNA-triggered innate immune responses.

(H) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2 and A3). At 24 h after transfection, the cells were infected with HSV-1 (MOI=1) for 8 h. Then the cells were lysed for real-time PCR assays.

The data are representative of three independent experiments and are presented as mean ± SD. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

Figure 3B

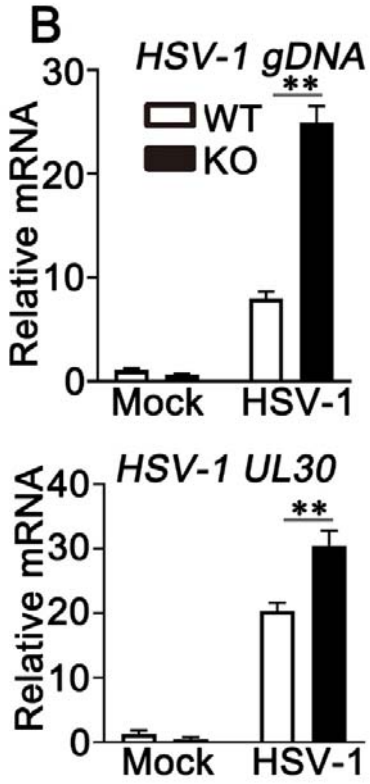


Figure 3 RNF144A deficiency impairs DNA virus or exogenous cytosolic DNA-triggered innate immune responses in BMDMs

(B) Wild-type (WT) and *Rnf144a*-deficient (KO) BMDMs were infected with HSV-1 (MOI=1) for 8 h. Then the cells were lysed for real-time PCR assays.

The data are representative of three independent experiments and are presented as mean ± SD. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

Figure 4B

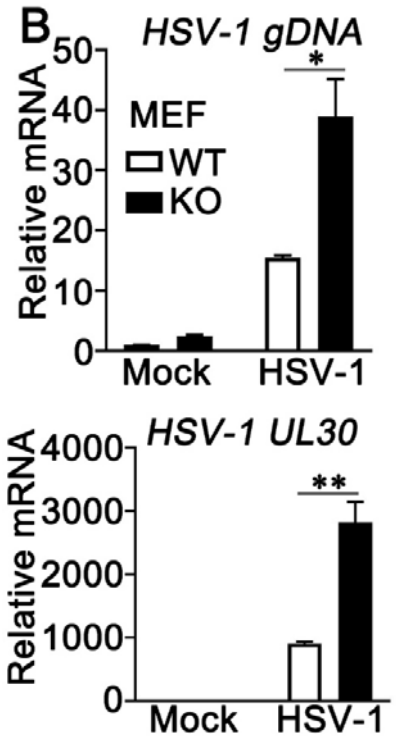


Figure 4 RNF144A deficiency impairs DNA virus- or exogenous cytosolic DNA-triggered innate immune responses in MEFs

(B) Wild-type (WT) and *Rnf144a*-deficient (KO) MEFs were infected with HSV-1 (MOI=1) for 8 h. Then the cells were lysed for real-time PCR assays.

The data are representative of three independent experiments and are presented as mean ± SD. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

5. In Fig2, the authors generate a RNF144A KO mouse model, but do not discuss any phenotype related to STING function. It would be interesting to see what the effects are of loss of STING in RNF144A KO cells. In F2E it remains unclear which organ was tested. Here, it also remains unclear if RNF144A affects viral uptake, replication or the formation of novel viral particles. The authors should carefully work these processes out in a systematic manner.

Response: This is a very good question. We explored what the effects are of loss of STING in RNF144A KO cells. As shown in Figure N7, in *Rnf144a*-deficient MEFs, STING

knockdown still caused a drop in the expression of IFN-β, CXCL10, and IFIT1 in response to HSV-1 infection, suggesting that although RNF144A increased STING-mediated signaling, STING still kept part of its function in the absence of RNF144A. In F2E, we labeled in the panels that the lung, liver, and spleen from the mice were tested. Additionally, in *STING*-deficient THP1 cells, RNF144A knockdown did not affect HSV-1-induced production of IFN-β, IP-10, ISG56, and IL-6 (Fig EV4K), and HSV-1 infection (Fig EV4K-M), indicating that the effect of RNF144A on HSV-1 infection was dependent on the existence of STING and innate immune responses, not by affecting virus directly.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

Figures EV4K-M

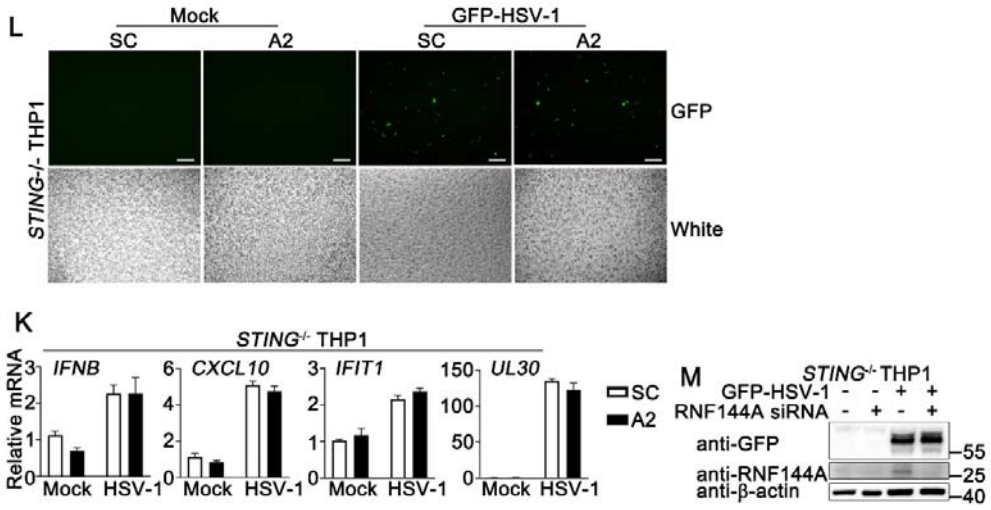


Figure EV4 RNF144A regulates STING-mediated signaling pathways.

(K) *STING*-deficient (KO) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with HSV-1 for 8 h. Afterward, the cells were lysed for real-time PCR assays.

(L, M) *STING*-deficient (KO) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with GFP-HSV-1 for 24 h. Afterward, the cells were subjected to image taken (L) or immunoblot assays (M).

β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

6. In Fig4, the authors should reconstitute their KO MEFs with WT RNF144A and the above-mentioned RNF144A mutants to really control their observations. The panel for F4J is missing.

Response: This is a very good question. In the previous version, we already reconstituted the KO MEFs with WT RNF144A and the above-mentioned RNF144A mutants in Figures 7B and 7C (Figures 6B and 6C in the revised version). Wild-type RNF144A and its mutants were transfected into RNF144A-deficient MEFs and the effect of these ligase-dead mutants was examined. As expected, the C20/23A and C198A mutants of RNF144A lost the ability to increase the ubiquitination of endogenous STING in MEFs (Fig 6B). Further, neither C20/23A nor C198A mutant of

RNF144A increased HSV60-induced production of IFN-β and IP-10 as wild-type RNF144A did, indicating the integrity of RING domain was required for the role of RNF144A in STING ubiquitination and STING-mediated signaling pathway (Fig 6C). Panel F4J (F4K in the revised version) was shown in the previous version in the middle right side of Figure 4.

Figures6B and 6C

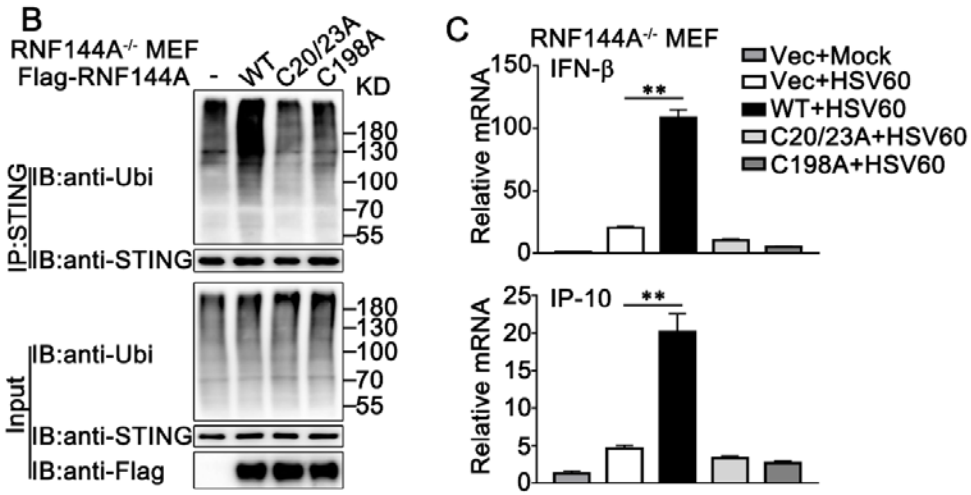


Figure 6 RNF144A promotes the ubiquitination of STING

(B) RNF144A-deficient (KO) MEFs were transfected with various combinations of plasmids as indicated. 24 h later, immunoprecipitation (IP) and immunoblot (IB) assays were performed.

(C) RNF144A-deficient (KO) MEFs were transfected with empty vector (Vec), wild-type RNF144A plasmid, or its mutants for 24 h and then transfected with HSV60 (1ug/ml) for 8 h. Then the cells were lysed for real-time PCR assays.

The data are representative of three independent experiments and are presented as mean ± SD. **, $p < 0.01$.

Figure 4K (Figure 4J in previous version)

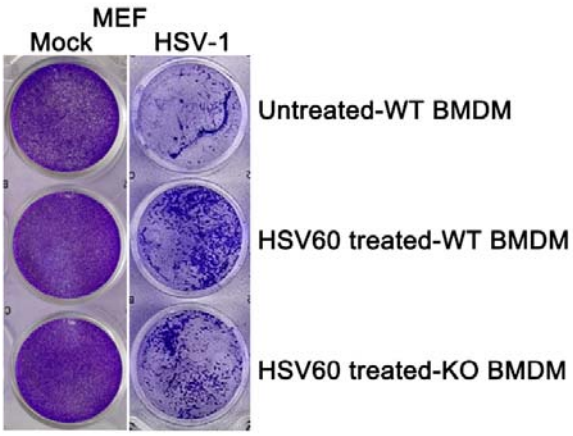


Figure 4 RNF144A deficiency impairs DNA virus- or exogenous cytosolic DNA-triggered innate immune responses in MEFs

(K) Wild-type (WT) and RNF144A-deficient (KO) BMDMs were transfected with mock or HSV60 (1ug/ml). Equal volumes of culture supernatants from these treatments were applied to fresh MEFs, followed by HSV-1 infection. The proliferation of cells was examined by crystal violet staining.

The data are representative of three independent experiments.

7. Panel F5E is not clear and does not show any difference between WT and KO.

Response: This is a very good question. As shown in Table N1, there is a difference between WT and KO in the expression of the genes shown in panel F5E.

Table for referee with unpublished data and its description has been removed upon request by the authors.

8. The authors describe an interaction between RNF144A and STING, but it remains unclear if this occurs endogenous. The authors should demonstrate more clearly that this interaction occurs without overexpression. In addition, the authors should demonstrate where in the cells this interaction occurs.

Response: This is a very good suggestion and has been well taken. In the previous version, Figures 6E and 6F showed the interaction between endogenous RNF144A and STING. Additionally, in this revised version, we added Figure EV5A to exhibit where this interaction occurs in the cells. As shown in Figure EV5A, upon HSV-1 infection, overexpressed RNF144A colocalized with endogenous STING mostly in the perinuclear vesicles. It will be better to use endogenous RNF144A in this assay. However, our antibody against RNF144A is not good enough for the confocal assay, so we used Flag-RNF144A instead.

Figure EV5A

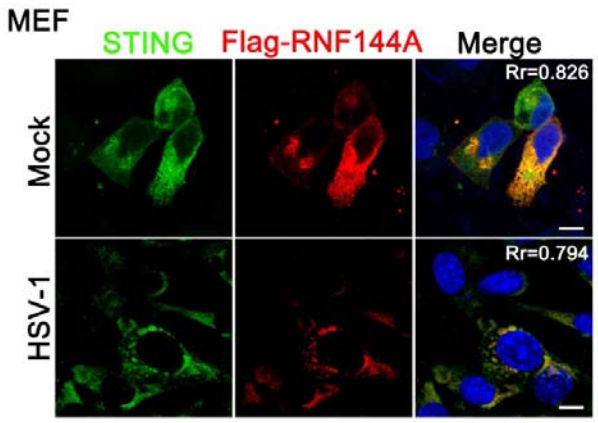


Figure EV5 RNF144A promotes the ubiquitination of STING.

(A) MEFs were transfected with expressing plasmids for Flag-RNF144A. At 24 h after transfection, MEFs were infected with HSV-1 (MOI=1) or left uninfected for 8 h. Immunofluorescence assays were performed using anti-STING (green) and anti-Flag (red). Nuclei were stained with DAPI. Scale bar: 10 μm. Pearson's correlation coefficient was calculated using ImageJ software. Rr, Pearson's correlation coefficient. The data are representative of three independent experiments.

9. The ubiquitination experiments are all done with overexpressed plasmids. The authors should show that STING ubiquitination at K236 by RNF114A happens endogenous by mass-spectrometric approaches.

Response: This is a very good suggestion and has been well taken. Other than the ubiquitination experiments with overexpressed plasmids, we also examined the endogenous ubiquitination in Figures 7D and EV5C in the previous version. Additionally, we added Figure 6D and EV5E to address this point further. Further, RNF144A knockdown in PMA-THP1 cells decreased HSV-1-induced ubiquitination of STING (Figure 6D). Next, we examined the effects of RNF144A on the ubiquitination of endogenous STING in MEFs, BMDMs, and PMs. Compared to control wild-type cells, RNF144A-deficient MEFs, BMDMs, or PMs displayed impairment in HSV-1-induced ubiquitination of STING (Figures 6E, EV5D and EV5E). The mass-spectrometric approach is a very good method to identify the modifications of

the target protein. However, besides RNF144A, several other proteins also target K236 of STING for ubiquitination and the ubiquitination of K236 of STING has been proved. Thus, we did not do the experiment in this version. But we will do it if the reviewer considers it is required.

Figure 6D

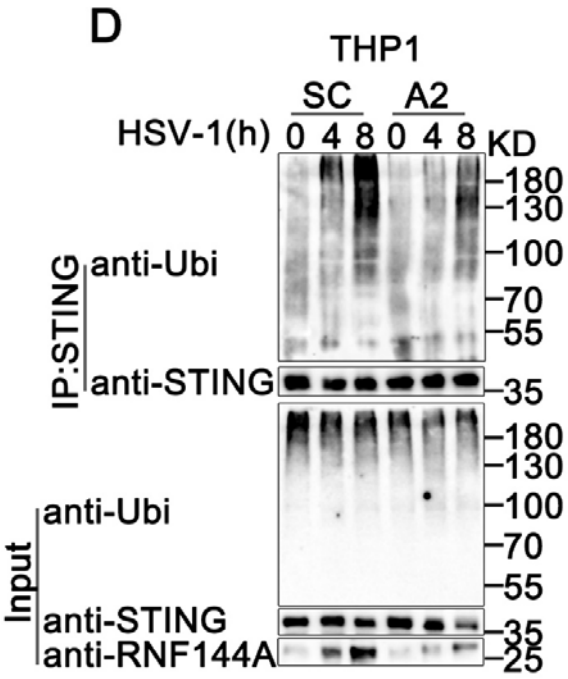


Figure 6 RNF144A promotes the ubiquitination of STING

(D) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2). At 24 h after transfection, the cells were infected with HSV-1 (MOI=1) for indicated periods. Afterward, immunoprecipitation (IP) and immunoblot (IB) assays were performed.

The data are representative of three independent experiments.

Figure EV5D and Figure EV5E

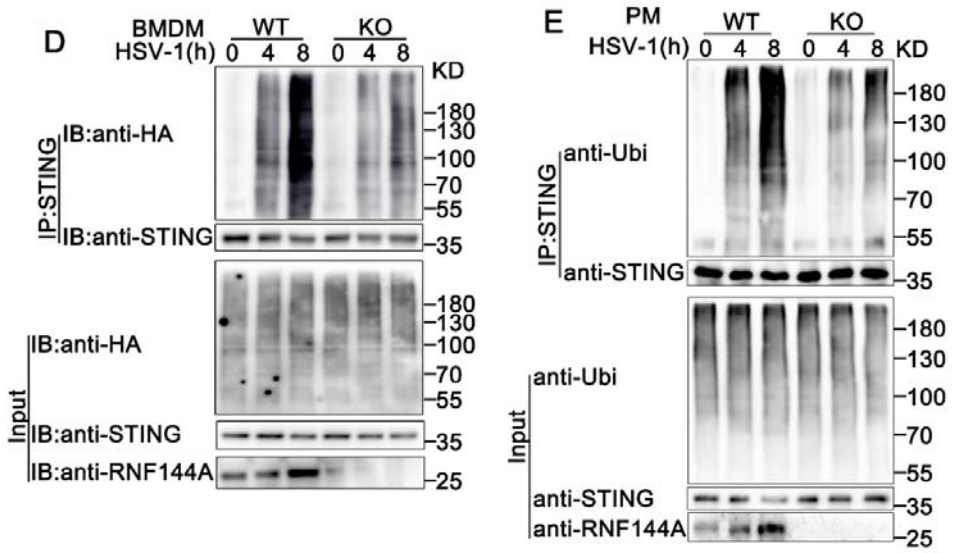


Figure EV5 RNF144A promotes the ubiquitination of STING.

(D, E) Wild-type (WT) and RNF144A-deficient (KO) BMDMs (D) or PMs (E) were infected with HSV-1 (MOI=1) for indicated periods. Afterward, the cells were lysed and subjected to immunoprecipitation (IP) and immunoblot (IB) assays.

The data are representative of three independent experiments.

10. The SLE experiments are interesting but stand alone with respect to the other findings. Connecting these findings with STING ubiquitination would be interesting and justify more why the authors especially looked into SLE.

Response: This is a very good question. However, STING ubiquitination assays require a large number of PBMCs, and it is not easy for us to collect enough PBMCs from SLE patients to perform the experiment. We especially looked into SLE because the roles of STING and type I IFN have been clarified to be very important in SLE (Hasan et al., 2015; Motwani et al., 2019).

11. It remains unclear if STING ubiquitination occurs directly by RNF144A or if it a consequence of more STING induced by RNF144A-mediated increases of IFNs during virus infection. The authors should address these effects by blocking auto- and

paracrine IFN signalling, and analyse RNF144A-mediate STING ubiquitination.

Response: This is a very good question. We used Fludarabine, the inhibitor of STAT1 activation, to inhibit IFN signaling. As shown in Figure N4, with the treatment of Fludarabine, RNF144A and STING could not be induced by HSV-1 infection. Then, we examined the ubiquitination of STING in the presence of Fludarabine and the results indicated that RNF144A deficiency decreased the ubiquitination of STING when the expression of STING exhibited no significant difference between wild-type and RNF144A-deficient cells (Figure N5). However, in the study of our lab and other groups, HSV-1 infection or viral DNA transfection did not increase STING expression, but decreased STING expression (PLoS Pathog. 2020 Mar 3, PLoS Pathog. 2015 Jun 26, Cell Discov. 2018 Mar 20). Further, our data did not suggest RNF144A affected STING expression, for example, in Figures 1J, 3G-H, 6D, 7A-B, 7D-H, and 8G-H. Thus, we did not put the Fludarabine data into our manuscript.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

12. The number of figures is too much and should be combined/condensed/restructured.

Response: This is a very good suggestion and has been well taken. Among the 11 figures in the previous version, Figure 11 is the working model; Figure 5 is the sequencing data; Figure 10 is the SLE results; all the other figures are relatively big and not easy to combine. So we put the previous Figures 5, 10, and 11 into the Appendix as Appendix Figures S1, S2, and S3.

Referee #3:

In this manuscript, the authors reported that RNF144A, an E3 ligase, K6-ubiquitinated STING and that this ubiquitination was essential for STING translocation from the ER to the Golgi. The results are potentially interesting, but several major issues need to be clarified.

Major critiques:

(1) This reviewer was concerned about the site of action of RNF144A. The authors suggested that the interference of RNF144A with cGAS was negligible (Fig. 6B and Fig. EV4A/B). The authors should also quantitate the amount of cGAMP in dsDNA-stimulated cells.

Response: The reviewer`s suggestion is very good and has been well taken. We examined the amount of cGAMP in HSV60- or HT DNA-stimulated MEFs and the results were shown in EV4A. In MEFs, after HSV-60 or HT DNA transfection, RNF144A deficiency did not make a significant difference in cGAMP generation (Fig EV4A).

Figure EV4A

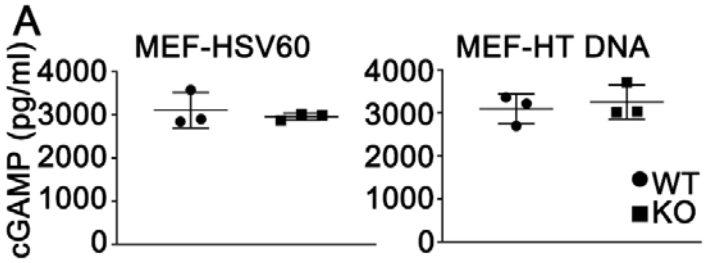


Figure EV4 RNF144A regulates STING-mediated signaling pathways.

(A) Wild-type (WT) and RNF144A-deficient (KO) MEFs were transfected with HSV60 (1μg/ml) or HT DNA (1μg/ml) for 24 h. Then the supernatants were collected and subjected to ELISA assays.

The data are representative of three independent experiments and are presented as

mean ± SD.

(2) All the imaging data should be quantitated with the Pearson co-efficient. It is extremely hard to interpret the data without quantitation. For example, In Fig. 8C, some of KO cells with infection of HSV-1, showed the good colocalization of STING with GM130.

Response: The reviewer`s suggestion is very good and has been well taken. We added the Pearson co-efficient quantification to all the colocalization results throughout the manuscript, including Figures 5H, 7A-C, 8I, and EV5A.

Figure 5H

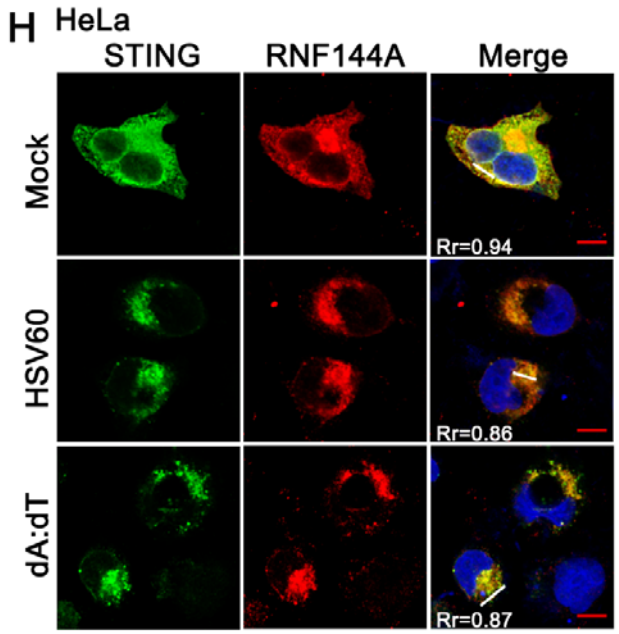


Figure 5 RNF144A interacts with STING

(H) HeLa cells were transfected with expressing plasmids for HA-STING and Flag-RNF144A. At 24 h after transfection, HeLa cells were transfected with HSV60 (1μg/ml), poly(dA:dT) (1μg/ml), or left untreated for 8 h. Immunofluorescence assays were performed using anti-HA (green) and anti-Flag (red). Nuclei were stained with DAPI. Plots of pixel intensity along the white line were shown in the right panel.

Pearson's correlation coefficient was calculated using ImageJ software. Rr, Pearson's correlation coefficient.

The data are representative of three independent experiments.

Figures 7A-C

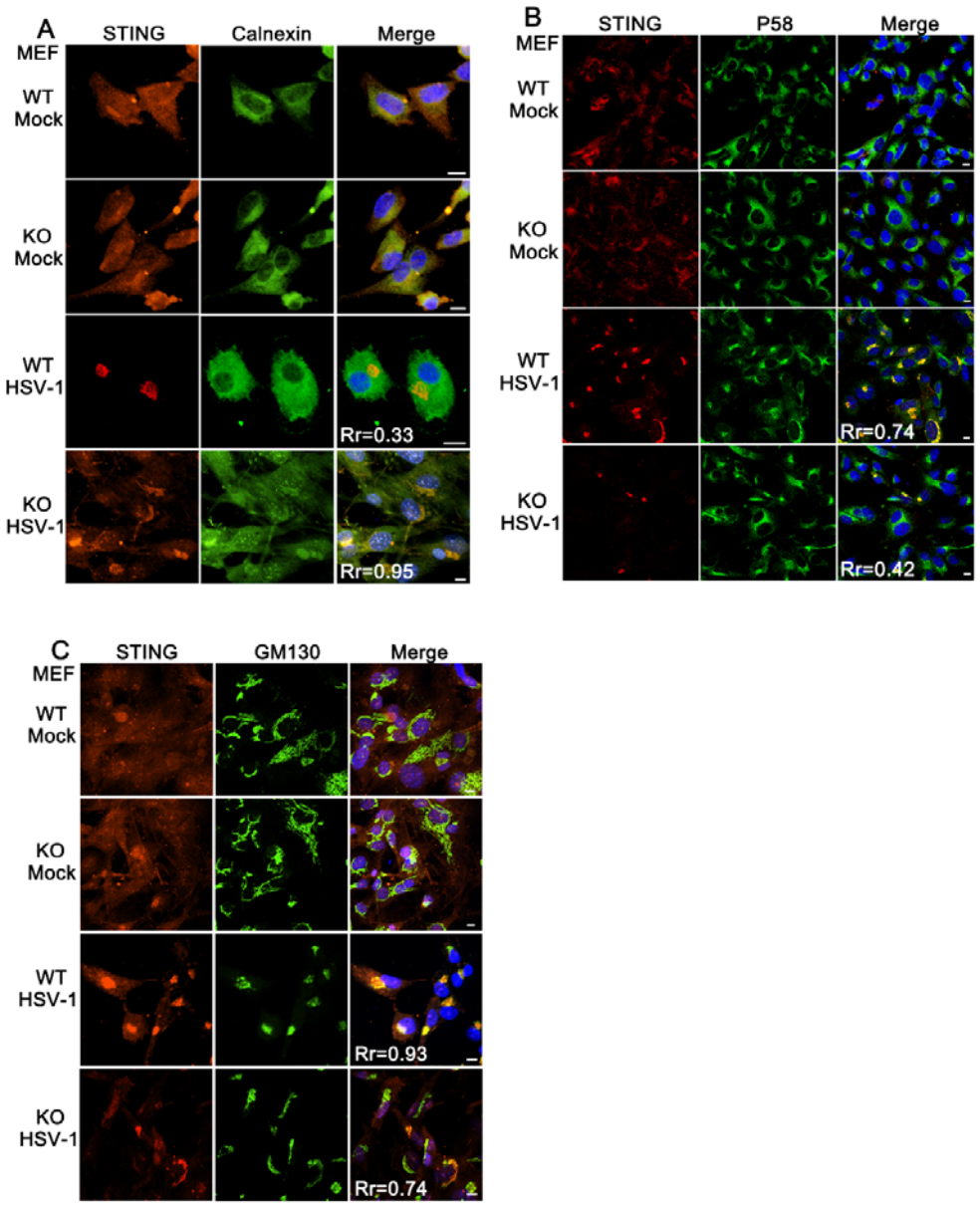


Figure 7 RNF144A deficiency impairs the translocation, dimerization, and complex formation of STING

(A-C) Wild-type (WT) and RNF144A-deficient (KO) MEFs were infected with HSV-1 (MOI=1) for 4 h. Afterward, the cells were fixed and labeled with STING (red) and

calnexin (ER marker, green) (A), P58 (ERGIC marker, green) (B), or GM130 (Golgi marker, green) (C) antibody. Scale bar: 10 μm. Pearson's correlation coefficient was calculated using ImageJ software. Rr, Pearson's correlation coefficient.

The data are representative of three independent experiments.

Figure 8I

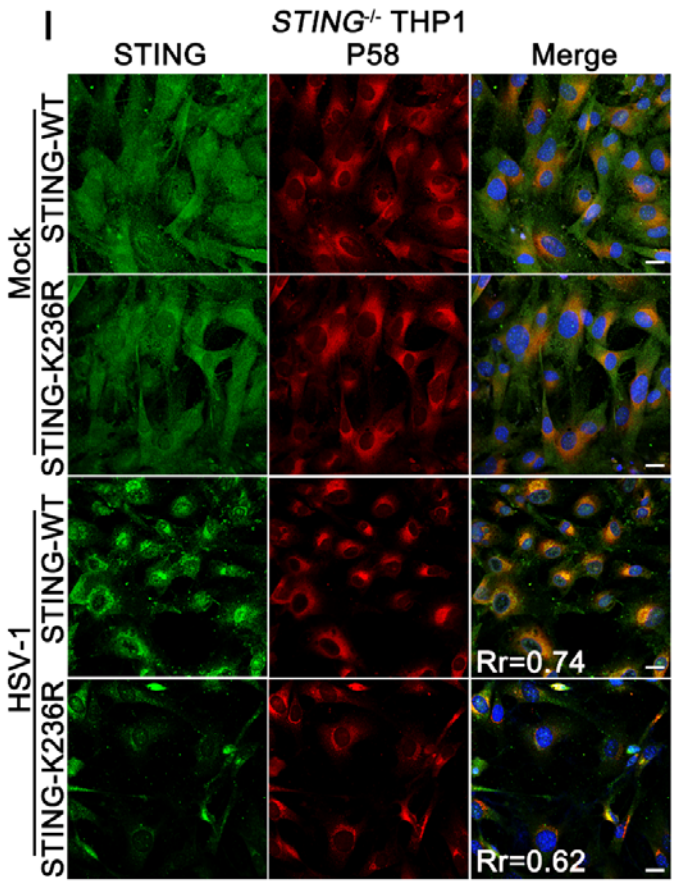


Figure 8 RNF144A promotes ubiquitination of STING at K236

(I) *STING*-deficient (KO) PMA-THP1 cells were overexpressed with the indicated plasmids. 24 h later, the cells were infected with HSV-1 (MOI=1) for 4 h. Afterward, the cells were fixed and labeled with STING (green) and P58 (ERGIC marker, red) antibodies. Scale bar: 10 μm. Pearson's correlation coefficient was calculated using ImageJ software. Rr, Pearson's correlation coefficient.

The data are representative of three independent experiments.

Figure EV5A

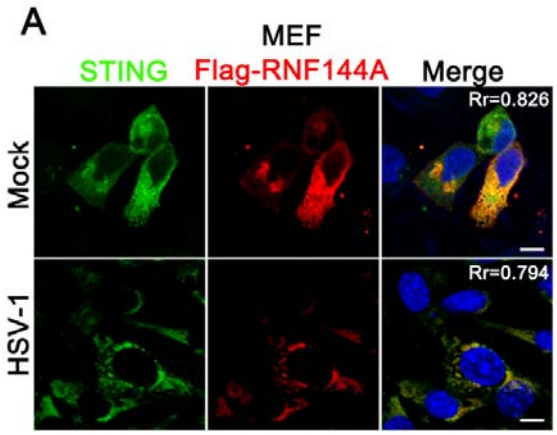


Figure EV5 RNF144A promotes the ubiquitination of STING.

(A) MEFs were transfected with expressing plasmids for Flag-RNF144A. At 24 h after transfection, MEFs were infected with HSV-1 (MOI=1), or left untreated for 8 h. Immunofluorescence assays were performed using anti-STING (green) and anti-Flag (red). Nuclei were stained with DAPI. Plots of pixel intensity along the white line were shown in the right panel. Pearson's correlation coefficient was calculated using ImageJ software. Rr, Pearson's correlation coefficient.

The data are representative of three independent experiments.

(3) The key WB data should be quantitated. For example, the difference of the intensity of pIRF3 between SC- or A2-treated cells (Fig. EV4A/B) appeared very subtle.

Response: The reviewer`s suggestion is very good and has been well taken. We added the quantification of the intensity of pIRF3, pTBK1, and pp65 in Figures EV4C, F, and I.

Figures EV4C, 4F, and 4I

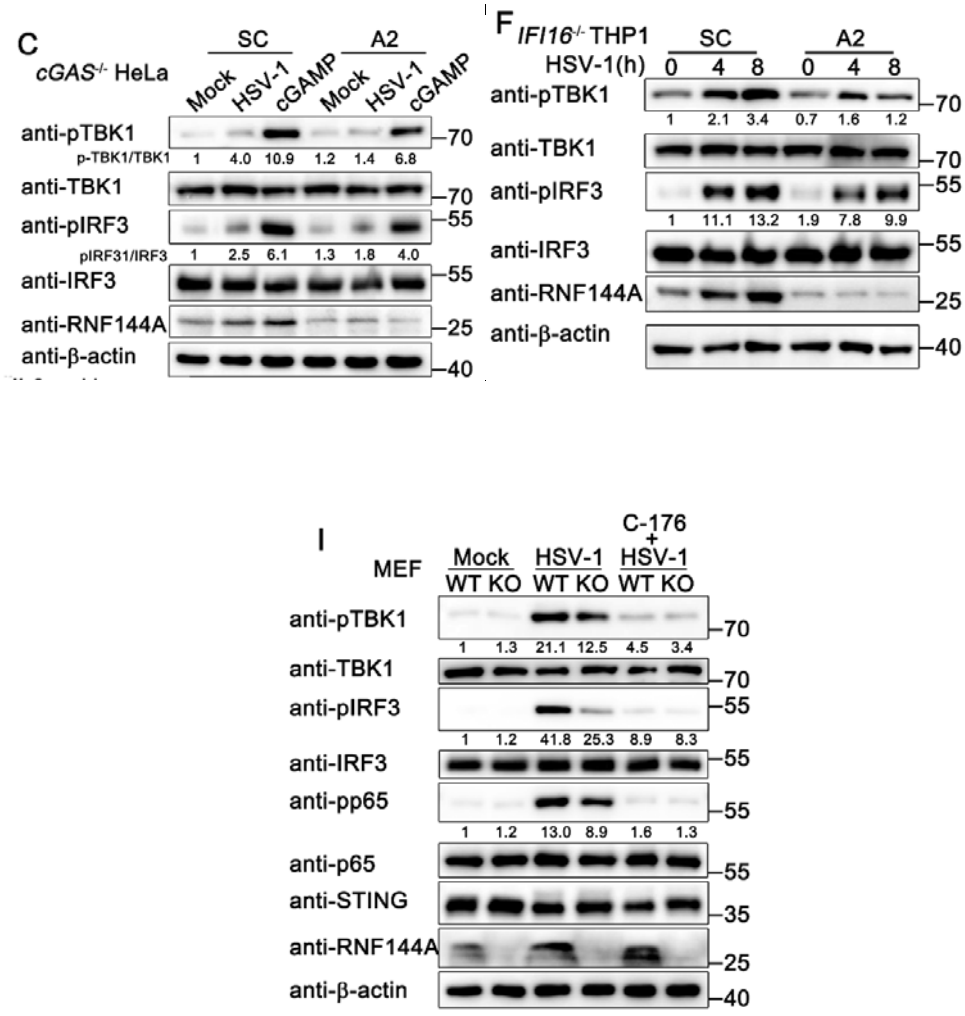


Figure EV4 RNF144A regulates STING-mediated signaling pathways.

(C) *cGAS*-deficient (KO) HeLa cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with HSV-1 or transfected with cGAMP (1μg/ml) for 8 h. The cells were lysed for immunoblot assays.

(F) *IFI16*-deficient (KO) PMA-THP1 cells were transfected with control siRNA (SC) or RNF144A-specific siRNA (A2) for 24 h and then infected with HSV-1 (MOI=1) for indicated periods. Afterward, the cells were lysed for immunoblot assays.

(I) Wild-type (WT) and *Rnf144a*-deficient (KO) MEFs were treated with C-176 (1μM) or left untreated for 24 h and then infected with HSV-1 (MOI=1) for 8 h. Afterward, the cells were lysed for immunoblot assays.

β-actin served as a loading control in all the immunoblot assays. The data are representative of three independent experiments.

(4) Regarding the regulation of STING trafficking out of the ER by ubiquitination, several reports have been published, as the authors discussed in Discussion. The apparent problem is that the same K236 residue with different Ub chain generated by different E3 ligases is suggested to play a role in the STING trafficking out of the ER. The authors should investigate and clarify the relationship between RNF144A, AMFR, and TRIM32.

Response: This is a very good question. It would be very interesting if the relationship between RNF144A, AMFR, and TRIM32 is clarified. Thus, we did various experiments to address this point. Firstly, we explored whether RNF144A interacted with AMFR or Trim32. As shown in Figure N1A, exogenously expressed RNF144A could co-immunoprecipitated with AMFR, but not Trim32, suggesting RNF144A might interact with AMFR. However, in HSV-1 infected THP1 cells, no endogenous interaction between RNF144A and AMFR or Trim32 was detected (Figure N1B), suggesting the exogenous interaction between RNF144A and AMFR might be artificial. Next, we examined whether RNF144A competed with AMFR or Trim32 for the interaction with STING. As shown in Figure N1C, we did not find RNF144A had a significant effect on the interaction between STING and AMFR or Trim32. Then, we investigated the effect of RNF144A on the function of AMFR and Trim32. HEK293T cells were transfected with cGAS and STING, together with indicated plasmids for 24 h, and IFN-β expression was evaluated by real-time PCR. As expected, RNF144A, AMFR, and Trim32 promoted cGAS-STING-induced IFN-β expression separately. However, RNF144A only promoted AMFR- or Trim32-induced IFN-β expression slightly, even not as strongly as RNF144A did by itself (Figure N1D). Then we explored whether the function of AMFR or Trim32 was dependent on RNF144A. Thus, we knocked down AMFR or Trim32 expression in *Rnf144a*-deficient MEFs and explored the effect of RNF144A deficiency on the function of AMFR or Trm32. The knockdown efficiency is shown in Figure N1E. Here, we noticed that, when AMFR or Trim32 was knocked down, RNF144A still had a strong effect on HSV-1-induced anti-viral immune

responses. However, in *Rnf144a*-deficient MEFs, AMFR or Trim32 knockdown only inhibited HSV-1-induced anti-viral immune responses very slightly. It seems that RNF144A might be required for the full function of AMFR and Trim32, but further study is required to draw a conclusion.

Additionally, Wang et al. reported that using the two-step immunoprecipitation assay, they failed to detect any ubiquitination signal of STING in the presence of Trim32 and they considered that Trim32 did not catalyze the polyubiquitination of STING per se but might promote the poly-ubiquitination of other proteins in the STING complex (Immunity 41, 919–933, December 18, 2014). In the same article, Wang et al. identified that other than K236, AMFR also promoted the ubiquitination of STING at K137, K150, and K224 (Immunity 41, 919–933, December 18, 2014). These results may suggest that Trim32 and AMFR do not have to compete with RNF144A for the modification of STING at K236 to exert their roles in signal transduction.

Taken together, till now, we did not find evidence strong enough to draw a conclusion about the relationship between RNF144A, AMFR, and Trim32. I think it may be better to address this question by using double- or triple-knockout mice. Considering we do not have such mice now and it will need a long period to generate these mice, we did not do these experiments, and we did not add these preliminary data to the manuscript. Instead, we revised the discussion about the K236, to avoid the misunderstanding that the K236 lysine residue of STING is the only target of Trim32 and AMFR.

The description of K236 was rewritten as “It has been reported that another two E3 ubiquitin ligases, autocrine motility factor receptor (AMFR) and TRIM32, also targeted STING for ubiquitination at lysine residues including K236. AMFR promoted the K27-linked polyubiquitination of STING, whereas TRIM32 catalyzed the K63-linked polyubiquitination of STING (Wang et al, 2014; Zhang et al, 2012). However, Wang et al. reported that using the two-step immunoprecipitation assay, they failed to detect any ubiquitination signal of STING in the presence of Trim32 and they considered that Trim32 did not catalyze the polyubiquitination of STING per se

but might promote the poly-ubiquitination of other proteins in the STING complex (Immunity 41, 919–933, December 18, 2014). Additionally, other than K236, AMFR also promoted the ubiquitination of STING at K137, K150, and K224 (Immunity 41, 919–933, December 18, 2014). These results may suggest that the modifications at K236 of STING are not exactly the same and redundant, but it remains unclear how the host cells organize these modifications on STING."

Figure for referee with unpublished data and its description has been removed upon request by the authors.

(5) The K6-ubiquination of STING by RNF144A occurred even in the presence of BFA?

Response: This is a very good suggestion and has been well taken. We added Figure EV5I to address this point. The answer is “Yes”. Further, the K6-linked ubiquitination of STING by RNF144A occurred even in the presence of BFA, indicating that the ubiquitination of STING by RNF144A was independent of STING translocation (Figure EV5I).

Figure EV5I

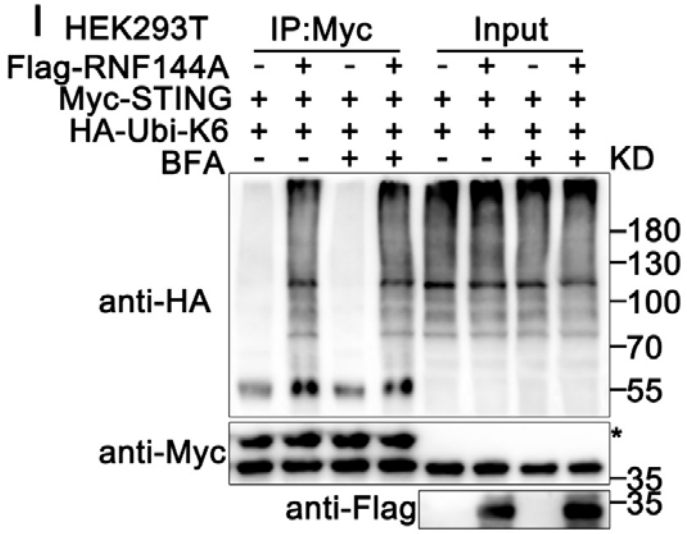


Figure EV5 RNF144A promotes the ubiquitination of STING and regulates its translocation.

(I) HEK293T cells were transfected with the indicated plasmids. At 24 h after transfection, were treated with BFA (5ug/ml) or left untreated for 3 h. Afterward, the cells were lysed and subjected to immunoprecipitation (IP) and immunoblot (IB) assays. * Heavy chain of the antibody.

The data are representative of three independent experiments.

Minor critiques:

(1) Introduction needs to address the precedent publications about STING ubiquitination and its involvement of STING trafficking, signalling, and degradation.

Response: The reviewer`s suggestion is very good and has been well taken. We added sentences as “Several E3 ubiquitin-protein ligases have been demonstrated in the regulation of STING trafficking, signaling, and degradation. For example, mitochondrial E3 ubiquitin protein ligase 1 (MUL1) catalyzes the ubiquitination of STING and facilitates optimal STING trafficking (Ni et al, 2017). TRIM32 and AMFR target STING for K63-linked or K27-linked ubiquitination separately and positively modulate type I IFN production (Wang et al, 2014; Zhang et al, 2012). Our previous

research indicated that RNF90 regulates antiviral responses by targeting STING for K48-linked ubiquitination and subsequent degradation after viral infection (Yang et al, 2020a). RNF5 and Trim30α also regulated STING ubiquitination and degradation (Wang et al, 2015; Zhong et al, 2009)."

(2) Konno et al. indicated that K224 ubiquitination by MUL1 was essential for STING trafficking (Sci Immunol 2017). At least, the report was cited and discussed.

Response: The reviewer`s comment is very good and has been well taken. In the discussion part, we discussed the K224 ubiquitination by MUL1 as "For example, MUL1 ubiquitinates STING on K224 via K63-linked polyubiquitination, which facilitates optimal STING trafficking and the transcription of host defense genes(Ni et al., 2017)."

(3) Some the data in main Figures can be transferred to suppelmentary Figures. For example, Figure 3 and 4 can be merged.

Response: This is a very good suggestion and has been well taken. We already put the data from PMs into EV2. If we put Figure 3 into supplementary Figures, there will be no data from immune cells in this part. If we put some panels in Figures 3 and 4 into supplementary Figures, it is not easy to number the panels because of the existence of EV2. Further, only up to 5 figures are allowed in Expanded View Figures. Among the 11 figures in the previous version, Figure 11 is the working model; Figure 5 is the sequencing data; Figure 10 is the SLE results; all the other figures are relatively big and not easy to combine. So we put the previous Figures 5, 10, and 11 into the Appendix as Appendix Figures S1, S2, and S3.

Dear Dr. Wang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of the study in EMBO reports. Referee #3 has some remaining concerns and suggestions to improve the study, I ask you to address in a final revised manuscript. Please also provide a final p-b-p-response for these.

Moreover, I have these editorial requests:

- I would suggest this simplified title:
RNF144A promotes antiviral responses by modulating STING ubiquitination

- Please provide the abstract written in present tense throughout.

- Please have your final manuscript text carefully proofread by a native speaker (also the legends).

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box in the submission system to provide more detailed descriptions and do NOT provide your final manuscript text file with an author contributions section. See also our guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section (DAS) - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please provide the Appendix file with page numbers and a proper table of contents (TOC) with page numbers.

- 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. There are several panels with diagrams without statistics. Please check. In particular:

Please indicate the statistical test used for data analysis in the legends of figures 1c-d, f-i; 2b, e-h; 3a-f; 4a-c, e-g; 6c; 8b, f, g; EV1a, b, e, f; EV2a-d; EV4e, g, h; EV5h.

Please define the annotated p values *** in the legend of figure 8f, g.

Please define the error bars in the legend of figures 5a-b.

I would suggest adding to each legend a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales. See:

https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please add scale bars of similar style and thickness to the microscopic images (main and EV figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, some scale bars are hard to see. Please check.

- Please add "Data ref:" as prefix to the data callouts in the text for the data citations (GSE37356; GSE45291). Please see:

https://www.embopress.org/page/journal/14693178/authorguide#datacitation

- Please explain in the legend for Fig. EV4L what 'White' means.

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.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have addressed all my concerns. I have no further comments.

Referee #2:

The authors have addressed the issues raised by this reviewer.

Referee #3:

In the revised manuscript, the authors addressed to my original concerns adequately. I have a few more suggestions to amend the manuscript.

(1) As shown in Figure N1F (in the response letter), the authors investigated the contribution of three E3 ligases (Trim32, AMFR, and RNF144A) into STING activation upon HSV-1 infection. The data are valuable in not only confirming the role of AMFR and Trim32 as published before, but suggesting that "these three are all required for the STING activation". I suggest putting Figure N1F into Discussion section in the revised manuscript with a concise conclusion.

(2) In the revised Figure 7B, the image of STING in cells (KO/HSV-1) appeared very different to the corresponding images in Figure 7A/C. The image is too dim and only a few puncta are recognizable. The image should be replaced, or the intensity should be increased.

Referee #3:

In the revised manuscript, the authors addressed to my original concerns adequately. I have a few more suggestions to amend the manuscript.

(1) As shown in Figure N1F (in the response letter), the authors investigated the contribution of three E3 ligases (Trim32, AMFR, and RNF144A) into STING activation upon HSV-1 infection. The data are valuable in not only confirming the role of AMFR and Trim32 as published before, but suggesting that "these three are all required for the STING activation". I suggest putting Figure N1F into Discussion section in the revised manuscript with a concise conclusion.

Responses: The reviewer`s suggestion is very good and has been well taken. We incorporated Figure N1F into the Discussion section as Appendix Figure S3, and we present our discussion of the data as follows.

Indeed, it would be fascinating to clarify the relationship between RNF144A, AMFR, and TRIM32. However, despite the ability of exogenously expressed RNF144A to co-immunoprecipitate with AMFR (Appendix Fig S3A), in HSV-1-infected THP1 cells, no endogenous interaction between RNF144A and AMFR or TRIM32 was observed (Appendix Fig S3B), suggesting that the exogenous interaction between RNF144A and AMFR may be artificial. Further, we did not observe a significant effect of RNF144A on the interaction between STING and AMFR or TRIM32 (Appendix Fig S3C). When transfected with cGAS and STING, RNF144A, AMFR, and Trim32 promoted cGAS-STING-induced IFN-β expression independently (Appendix Fig S3D). However, RNF144A only slightly enhanced AMFR- or TRIM32-induced IFN-β expression, and not as effectively as RNF144A did by itself (Appendix Fig S3D). When AMFR or Trim32 was knocked down, RNF144A still had a strong impact on HSV-1-induced anti-viral immune responses (Appendix Fig S3E and S3F). However, in Rnf144a-deficient MEFs, AMFR or Trim32 knockdown only slightly inhibited HSV-1-induced anti-viral immune responses (Appendix Fig S3F), indicating that RNF144A might be required for the full

function of AMFR and Trim32, but further studies are needed to draw a conclusion.

In addition, Wang et al. reported that using the two-step immunoprecipitation assay, they failed to detect any ubiquitination signal of STING in the presence of Trim32 and they considered that Trim32 did not catalyze the polyubiquitination of STING per se but might promote the poly-ubiquitination of other proteins in the STING complex (Wang et al., 2014). In the same article, Wang et al. identified that other than K236, AMFR also promoted the ubiquitination of STING at K137, K150, and K224 (Wang et al., 2014). These results may suggest that Trim32 and AMFR do not have to compete with RNF144A for the modification of STING at K236 to exert their roles in signal transduction. In summary, our current findings indicate that these three molecules are all necessary for STING activation, but may function through distinct mechanisms.

Appendix Fig S3

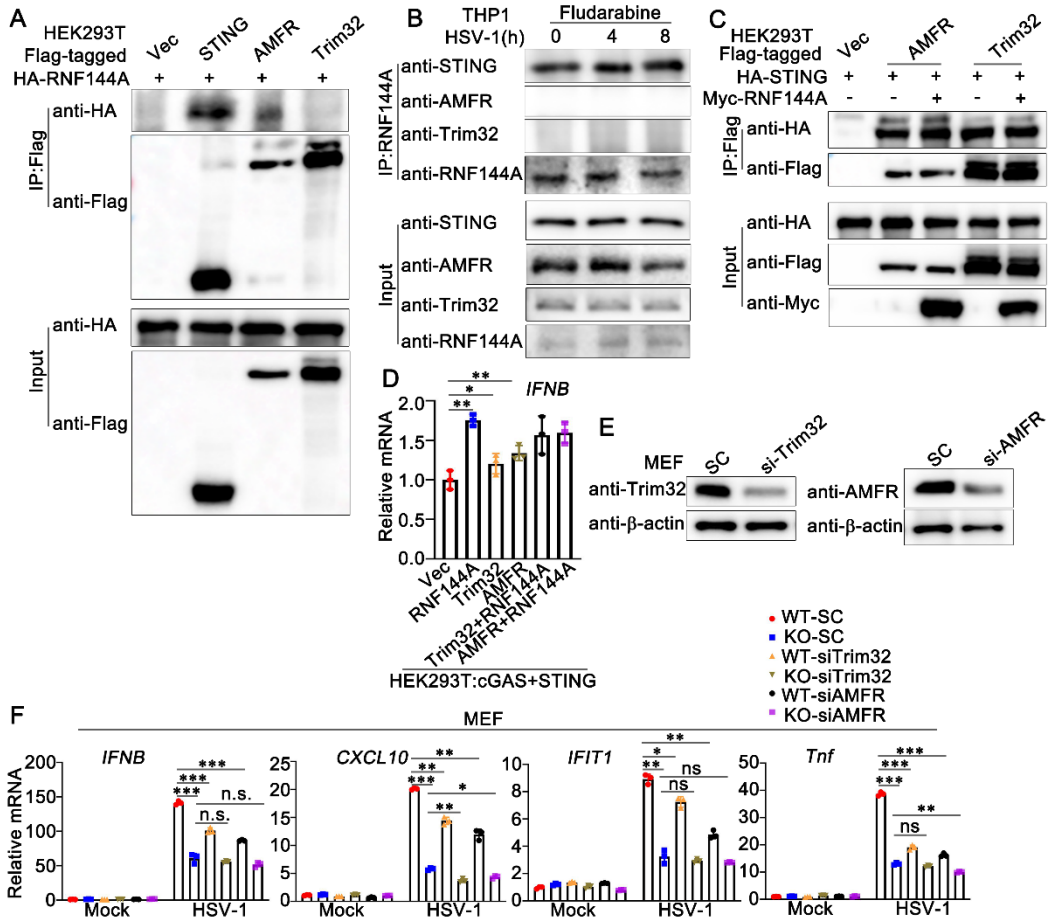


Figure S3 The relationship between RNF144A, AMFR, and Trim32.

A) HEK293T cells were transfected with indicated plasmids. At 24 h after transfection, immunoprecipitation (IP) and immunoblot (IB) assays were performed as indicated.

B) PMA-THP1 cells were treated with Fludarabine, and infected with HSV-1 for 0, 4, 8 h. The immunoprecipitation (IP) and immunoblot (IB) assays were performed as indicated.

C) HEK293T cells were transfected with the indicated plasmids. At 24 h after transfection, immunoprecipitation (IP) and immunoblot (IB) assays were performed as indicated.

D) HEK293T cells were transfected with indicated plasmids. At 24 h after transfection, IFN-β expression was detected by real-time PCR assays.

E) MEFs were transfected with control siRNA (SC), Trim32-specific siRNA (si-Trim32), or AMFR-specific siRNA (si-AMFR). At 24 h after transfection, the immunoblot assays were performed as indicated.

F) MEFs were transfected with control siRNA (SC), Trim32-specific siRNA (si-Trim32), or AMFR-specific siRNA (si-AMFR). At 24 h after transfection, the cells were infected with HSV-1 (MOI=1) for 8 h and then the real-time PCR assays were performed as indicated. Data information：*p < 0.05, **p < 0.01, ***p < 0.001 and n.s., not significant (p > 0.05); p values are calculated using two-tailed unpaired Student’s t test. Data are representative of at least three independent biological replicates. In (D, F), each data point represents a technical replicate. Error bars are presented as mean ± SD. Source data for this figure are available online.

(2) In the revised Figure 7B, the image of STING in cells (KO/HSV-1) appeared very different to the corresponding images in Figure 7A/C. The image is too dim and only a few puncta are recognizable. The image should be replaced, or the intensity should be increased.

Response: The reviewer`s suggestion is very good and has been well taken. The

image was replaced by a better one.

Figure 7B

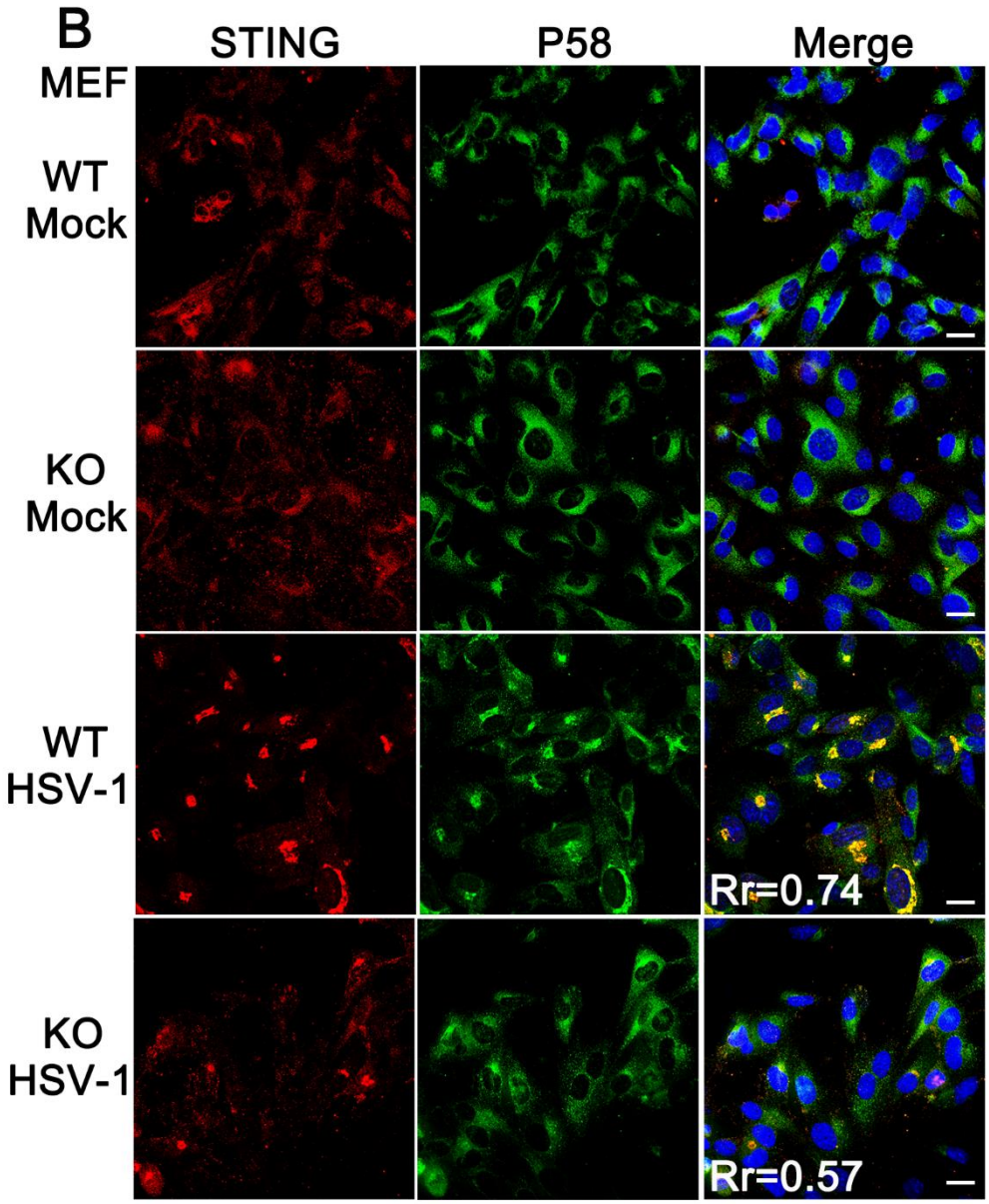


Figure 7 RNF144A deficiency impairs the translocation, dimerization, and complex formation of STING

(B) Wild-type (WT) and Rnf144a-deficient (KO) MEFs were infected with HSV-1 (MOI=1) for 4 h. Afterward, the cells were fixed and labeled with STING (red) and P58 (ERGIC marker, green) antibody. Scale bar: 10 μm (A, C); 20 μm (B). Pearson's correlation coefficient was calculated using ImageJ software. Rr, Pearson's correlation coefficient.

Editorial requests:

- I would suggest this simplified title:
RNF144A promotes antiviral responses by modulating STING ubiquitination

Responses: Agree. We have revised the title as suggested.

- Please provide the abstract written in present tense throughout.

Responses: Done. We have revised the abstract as suggested.

- Please have your final manuscript text carefully proofread by a native speaker (also the legends).

Responses: Done. The final manuscript have been revised by a native speaker.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box in the submission system to provide more detailed descriptions and do NOT provide your final manuscript text file with an author contributions section. See also our guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

Responses: Done. We have removed the author contributions section from the manuscript.

- Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section (DAS) - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure

legends

Responses: Done. We have ordered the manuscript sections using these names.

- Please provide the Appendix file with page numbers and a proper table of contents (TOC) with page numbers.

Responses: Done. We have provide the Appendix file with page numbers and a proper table of contents (TOC) with page numbers according to the paper on the current issue of *EMBO Reports*.

- 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. There are several panels with diagrams without statistics. Please check. In particular:

Please indicate the statistical test used for data analysis in the legends of figures 1c-d, f-i; 2b, e-h; 3a-f; 4a-c, e-g; 6c; 8b, f, g; EV1a, b, e, f; EV2a-d; EV4e, g, h; EV5h.

Please define the annotated p values *** in the legend of figure 8f, g.

Please define the error bars in the legend of figures 5a-b.

I would suggest adding to each legend a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales. See:

https://www.embopress.org/page/journal/14693178/authorguide#figureformat

Responses: Done. We have revised all the figures and added to each legend a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales.

- Please add scale bars of similar style and thickness to the microscopic images (main and EV figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, some scale bars are hard to see. Please check.

Responses: Done. We have added scale bars of similar style and thickness to the microscopic images (main and EV figures), using clearly visible black or white bars.

- Please add "Data ref:" as prefix to the data callouts in the text for the data citations (GSE37356; GSE45291). Please see:

https://www.embopress.org/page/journal/14693178/authorguide#datacitation

Responses: Done. We have added "Data ref:" as prefix to the data callouts in the text for the data citations (GSE37356; GSE45291).

- Please explain in the legend for Fig. EV4L what 'White' means.

Responses: Done. 'White' means 'Bright field microscopy'.

In addition, I would need from you:

- a short, two-sentence summary of the manuscript (not more than 35 words).

Responses: RBR family E3 ubiquitin ligase RNF144A promotes DNA virus- or cytosolic DNA-triggered signaling. RNF144A interacts with STING and increases its K6-linked ubiquitination, leading to the enhancement of STING translocation from the ER to the Golgi.

- two to four short (!) bullet points highlighting the key findings of your study (two lines each).

Highlights:

1 RNF144A is induced by HSV-1 infection and prevents mice from HSV-1 infection.

2 *Rnf144a*-deficient immune and non-immune cells exhibit impaired DNA virus- or cytosolic DNA-triggered signaling.

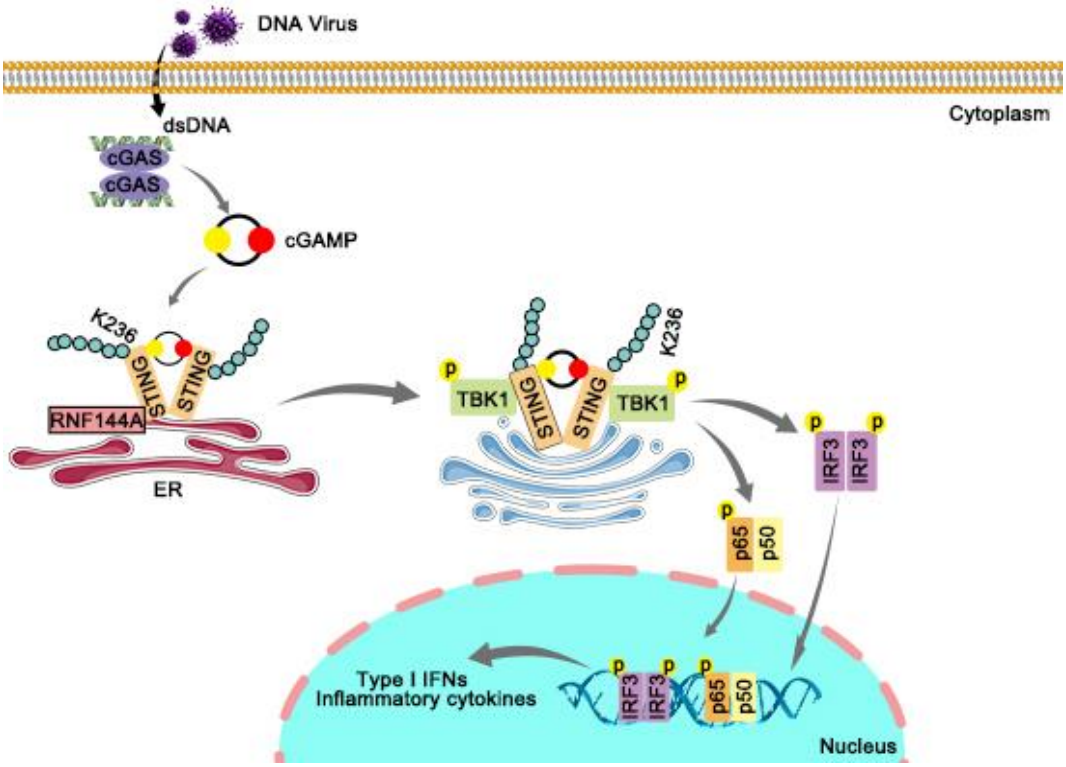
3 RNF144A interacts with STING and promotes K6-linked ubiquitination of STING at K236.

4 RNF144A is required for the translocation of STING from the ER to the Golgi in response to HSV-1 infection.

- 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.

Responses: Appendix Fig S4 is a schematic summary figure that provides a sketch of the major findings, only much bigger. We provided the figure with the exact size and

we will cancel the Appendix Fig S4 if needed.



Dr. Jie Wang
Xinxiang Medical University, Xinxiang
601 Jinsui Road
Xinxiang, Henan 453003
China

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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.

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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?	Yes	Page 30-31 Materials and Methods

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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	Page 31 Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table S1

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Page 31-32 Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Page 32 Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Page 30 Materials and Methods

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Page 34 Materials and Methods

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

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If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
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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	
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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Page 37-60 Figure legends

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Page 37-60 Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Page 37-60 Figure legends

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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.	**Yes**	Page 30 Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	**Yes**	Page 30 Materials and Methods
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	**Yes**	Page61-62 Reference list