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Positive feedback regulation of type I interferon by the interferon-stimulated gene STING

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Transaction Report:

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

Editor: Nonia Pariente

1st Editorial Decision 26 August 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although the referees find the topic of interest, they also point out the limited novelty of the findings and raise numerous serious concerns regarding the conclusiveness of the data and its biological relevance.

As the reports are below, I will not detail them here. However, it is clear that publication of your study in our journal cannot be considered at this stage and substantial additional experimental work would be required to sufficiently strengthen the work. Given the constructive reports provided and the guidance they afford on how to strengthen the study, I would like to invite its revision. However, please note that addressing the referee concerns in full would be a pre-requisite for publication in EMBO reports.

It is our policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.
Please note that a revised version would thus only be sent back to the referees if it fully addresses the referee concerns. I appreciate that experimentally addressing all the issues raised would involve extensive additional work of uncertain outcome and I would be willing to reasonably extend our normal 3 months for revision. However, I would also understand if you rather chose to seek rapid publication elsewhere.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. When submitting your revised manuscript, please also include editable TIFF or EPS-formatted figure files, a separate PDF file of any Supplementary information (in its final format) and a letter detailing your responses to the referees. Please do not worry about the manuscript length, as we have recently decided to publish full-length articles in addition to short reports. This should allow for the rewriting and discussion requested by referee 3. However, we do ask that you write succinctly and keep the text as short as possible.

Please contact me if I can be of any assistance during the revision process.

REFEREE REPORTS:

Referee #1:

Accumulating evidence shows that cGAS-STING signaling axis mediates a host defense response to DNA viruses by inducing expression of type I IFN and IFN-inducible genes. In this study, Cheng G and colleagues attempted to reveal a molecular mechanism of the positive feedback regulation of cGAS-STING signaling axis. Main findings were as follows: (1) STING is transcriptionally induced by cyclic dinucleotides (CDNs) through an IFNAR1 and STAT1 dependent pathway; (2) the promoter region of STING gene harbors functional STAT1 responsible sites; (3) ectopic expression of STING enables activation of cGAS-STING signaling axis in IFN receptor-deficient cells. This reviewer feels the present study interesting and valuable. The manuscript was well written. However, additional data is needed to strengthen the author's conclusion. The comments are shown below.

(1) In Fig.1F, the authors should show level of STING at time zero.
(2) In Fig.1 and Fig.S1, the authors showed MyD88-dependent suppression of STING expression. After the phosphorylation, STAT1 moves into nucleus and binding to the promoter region of IFN-inducible genes. Which step of STAT1 activation is inhibited by MyD88 signaling axis?
(3) In Fig.2, electrophoresis mobility shift assay and chromatin immunoprecipitation assay should be done to provide evidence that STAT1 directly binds with the promoter region of STING gene.
(4) In Fig.2C, the authors should examine effect of STAT1 expression on STING promoter-dependent expression of luciferase.
(5) In Fig.3, the authors should show level of IRF7, an IFN-inducible gene, and examine whether IRF7 is involved in STING-mediated expression of IFN-beta or not.
(6) In Fig.4B, the authors should examine production of IFN-beta by unstimuated J2 BMM.
(7) In Fig.4, the authors should compare level of STING between WT BMM stimulated with cGAMP and J2-BMM transduced with LV-STING. If level of STING in J2-BMM is much higher than that in WT BMM, the authors cannot conclude that induction of STING plays a role in IFN-mediated positive feedback loop of cGAS-STING signaling axis.

Referee #2:

In this work Ma et al propose that STING in an IFN-stimulated gene and that this contributes to stimulate positive feed-back for IFN induction. The issue of positive feed-back loops in regulation of IFN expression is well established, most notably through the classical work by Marie and Levy (EMBO J. 1998 Nov 16;17:6660-9.), and the implications of this phenomenon for in vivo biology have been demonstrated several times. The work presented is generally of high quality and the conclusions drawn are supported by the data. Despite this, does this reviewer find that the authors do not provide convincing evidence on the importance of STING induction in stimulation of IFN expression during infection. Even more importantly, the authors make no attempts to relate the proposed positive feed-back loop to the well-established IRF3->IFNb->IRF5->IFNa/b loop.

1. Figure 1. Data on mRNA levels of IRF3 and IRF7 in WT cells have to be provided.
2. Figure 1. STING mRNA induction in IRF7 KO cells should be tested to evaluate whether the

proposed mechanism is overlapping with the classical IFN positive feed-back look involving IRF7 induction.
3. Figure 1. The data would gain significantly if data were included from primary human cells and/or on IFNAR-dependent STING induction after infection with a relevant pathogen.
4. Figure 1F. It is surprising that the authors have not included data from untreated cells. It is well established that IFNAR-/- cells have a lower basal expression of ISGs. Therefore, it is central that data from untreated WT and IFNAR -/- cells are included.
5. Figure 2C. Can the authors demonstrate that IFN treatment of HEK293T cells activates the reporter gene in a manner dependent on the STAT binding site?
6. Figure 4A and B. IRF7-/- cells should be transduced with LV-STING and IFNb levels should be determined in order to examine the role of the classical IRF3-IFNb-IRF7-IFNa/b pathway in the proposed pathway.
7. The model in Figure 4D, should also include classical positive feedback look described by Marie and Levy (EMBO J. 1998 Nov 16;17:6660-9.).
8. All figures. The authors focus their measurements in IFNb. It would be relevant also to include IFNa-species known to be involved in the later stages of the positive feed-back response.
MINOR POINTS
1. I did not manage to find the data on LPS-stimulated IFN induction (described together with Figure 1G). Should be included.

Referee #3:

August 8th 2014
Review
Manuscript: Positive feedback regulation of type I interferon by the interferon-stimulated gene STING
Journal: EMBO reports impact factor (7.58)

Overall Comments:
• There are no significant flaws in experimental design or in interpretation of results; findings are interesting though not at all surprising.
• However, the authors do not delineate the novel findings from those that have already been shown by other workers. They do not effectively emphasize the novelty their work (i.e. 1st to show STING is regulated transcriptionally).
• The manuscript is very poorly written.

Specific Comments:
Abstract Section
• 1st line of abstract section- sentence reads "Stimulator of interferon genes (STING) plays an important role in innate immune response to pathogenic cytoplasmic DNA"...should read "Stimulator of interferon genes (STING) plays an important role in the innate immune response to pathogenic cytoplasmic DNA".
• Last line in the abstract- "Thus, our study has demonstrated that STING is an interferon-stimulated gene (ISG) and its induction plays a role in IFN-I positive feedback loop." Should read..."Thus, our study has demonstrated that STING is an interferon-stimulated gene (ISG) and its induction plays a role in the IFN-I positive feedback loop."

Introduction Section
• Last line of the 1st paragraph- sentence reads "Although STING-dependent IFN-I pathway has been extensively studied, it is largely unknown how to regulate STING itself, particularly at the transcriptional level."...should read..."Although the STING-dependent IFN-I pathway has been extensively studied, it is largely unknown how STING is regulated, particularly at the transcriptional level."
• 3rd sentence in the second paragraph- sentence reads "For example, induction of cytosolic RNA and DNA sensors such as RIG-I, MDA5, and IFI16 enhances the induction of IFN-I by sensing more pathogen nucleic acids." ...Sentence should read..."For example, induction of cytosolic RNA and DNA sensors such as RIG-I, MDA5, and IFI16 enhances the induction of IFN-I by sensing more pathogen-derived nucleic acids."

• 1st line of the last paragraph of the introduction- line reads "Here, our data indicate that STING could be induced by IFN-I via a STAT1 binding site in its promoter region; positive feedback regulation loop is required for optimal production of CDNs-triggered IFN-I." should read..."Here, our data indicate that STING expression could be induced by IFN-I via a STAT1 binding site in its promoter region; positive feedback regulation loop is required for optimal production of CDNs-triggered IFN-I."

Results and Discussion Sections
• 2nd sentence in the first paragraph- sentence reads "However, other genes in the downstream of STING signaling pathway..." sentence should read "However, other genes downstream of the STING signaling pathway..."
• Rationale and experiment for fig 1C...Is it not well established that STING is an ISG?
• I would have liked to have seen controls where STING expression was inhibited by shRNAs or through genetic deletion.
• 3rd line at the end of the 1st paragraph- sentence reads "Taken together, we have demonstrated that STING transcripts could be induced by IFN-1 and most of the ligands which could trigger IFN-1 in BMMs, therefore demonstrating that STING is an ISG." ...sentence should read "Taken together, we have demonstrated that STING transcripts could be induced by IFN-1 and most of the ligands which could trigger IFN-1 in BMMs, therefore demonstrating that STING is an ISG."
o Again, I do not think this is a novel claim.
• 3rd paragraph- sentence reads "Among all predicted TF binding sites around Sting transcription start site (TSS)..." sentence should read "Among all predicted TF binding sites around the Sting transcription start site (TSS)..."
• 4th paragraph- sentence reads "Both sites got high matrix score in the TRANSFAC software..." sentence should read "Both sites got high matrix scores in the TRANSFAC software..."
• 7th paragraph- sentence reads "...we hypothesized that the induction of STING could increase the host ability of sensing CDNs, and positively regulates CDN-triggered IFN-I production." Sentence should read "...we hypothesized that the induction of STING could increase the host's ability of sensing CDNs, and positively regulates CDN-triggered IFN-I production."
• The "discussion" is non-existent!! What do you think the novel mechanism of post-transcriptional regulation of STING-dependent type I interferon production is uniquely contributing to the host innate immune response??? Any speculations?? Probably extremely important to regulate the production of type I interferon during the host response because the over production can lead to severe inflammation and tissue damage. Because of this having multiple regulatory mechanisms is key!

The figures and figure legends
• Figure 1- the letters of each figure need to be aligned and graphs as well ...sloppy
• Figure 2- same comment from figure

1st Revision - authors' response 26 November 2014

Point to point response

Response to Reviewer #1:
Accumulating evidence shows that cGAS-STING signaling axis mediates a host defense response to DNA viruses by inducing expression of type I IFN and IFN-inducible genes. In this study, Cheng G and colleagues attempted to reveal a molecular mechanism of the positive feedback regulation of cGAS-STING signaling axis. Main findings were as follows: (1) STING is transcriptionally induced by cyclic dinucleotides (CDNs) through an IFNAR1 and STAT1 dependent pathway; (2) the promoter region of STING gene harbors functional STAT1 responsible sites; (3) ectopic expression of STING enables activation of cGAS-STING signaling axis in IFN receptor-deficient cells. This reviewer feels the present study interesting and valuable. The manuscript was well written. However, additional data is needed to strengthen the author's conclusion. The comments are shown below.

Response: Thanks for your comments. To address your concerns, we have added new data (**Fig 1H, 2C, 2D, 2H, 4A-F, 5B, and Supplementary Fig S2A, S4A**) and discussed in our revised manuscript (the changes have been yellow highlighted). We have provided point to point responses to your comments as below.

(1) In Fig.1F, the authors should show level of STING at time zero.
Response: We repeated this experiment and added the data of the time point "0" in our new figure. As you see in **Fig 1H** of our revised manuscript, 1) protein level of STING is comparable between inactivated WT and *Ifnar1-/-* BMMs (time point "0"); 2) STING is induced by cGAMP at 12h and 24h post transfection in WT BMMs; 3) Transfection of cGAMP downregulates STING protein level in *Ifnar1-/-* BMMs, which is an opposite phenotype in WT BMMs; 4) Significant higher STING level in cGAMP-activated WT BMMs than *Ifnar1-/-* BMMs.

As we described in this study, STING is an ISG, therefore it is reasonable that cGAMP could induce STING protein expression by triggering IFN-I production in WT BMMs. Given that STING could not be induced by cGAMP in *Ifnar1-/-* BMMs, it also makes sense that higher STING level was observed in cGAMP-activated WT BMMs than *Ifnar1-/-* BMMs. Interestingly, STING expression is downregulated by transfection of cGAMP in *Ifnar1-/-* BMMs. We don't know how STING is negatively regulated by cGAMP in the absence of IFNAR signaling in BMMs by now, maybe transcriptionally, post-transcriptionally (e.g. RNA binding protein-mediated mRNA degradation or miRNA-mediated translation inhibition), or posttranslational (protein degradation). It seems that regulation of STING is much more complicated than "STING is an ISG". Indeed, previous studies have shown that cGAMP destabilizes STING protein by triggering ULK1 phosphorylation in primary MEF and hTERT-BJ1 cells (PMID: **24119841**), which indicate that cGAMP may play different roles in regulating STING expression. Probably in WT BMMs, activation of BMM by cGAMP triggers both positive feedback via type I IFN production and negative feedback through other pathways such as AMPK-ULK1 pathway. Further studies are required for fully understanding how STING is fine regulated at multiple levels during innate immune responses.

(2) In Fig.1 and Fig.S1, the authors showed MyD88-dependent suppression of STING expression. After the phosphorylation, STAT1 moves into nucleus and binding to the promoter region of IFN-inducible genes. Which step of STAT1 activation is inhibited by MyD88 signaling axis?
Response: Based on our preliminary data, inhibition of STING by MyD88-depenent pathway happens earlier than the induction of STING through TRIF-dependent pathway in LPS-triggered macrophage. We have noticed: 1) LPS could suppress STING expression within 0.5h treatment (there is almost no detectable induction of IFN-I in these BMMs yet); 2) Suppression of STING by LPS does not require new protein synthesis (cycloheximide treatment doesn't affect the suppression). According to our results, we are proposing a model that activation of MyD88 by LPS (or TLR2 ligands) suppresses STING transcription by recruiting repressor to the promoter of STING at the early stage, following induction of IFN-I by LPS via TRIF-dependent pathway phosphorylates STAT1 and replaces that repressor with activator in STING promoter, and thus drive STING transcription. As we shown in **Fig 1J and Fig S1B** of our revised manuscript, STING mRNA was downregulated 4h post LPS stimulation and restored 12h post LPS stimulation, which partially supports our proposed model above. However, we have not got enough solid data to support this model and the main point of this manuscript is "STING is an ISG", therefore, we didn't include these preliminary data and discuss in detail about the dual regulation of STING by LPS in this manuscript.

(3) In Fig.2, electrophoresis mobility shift assay and chromatin immunoprecipitation assay should be done to provide evidence that STAT1 directly binds with the promoter region of STING gene.
Response: Thanks for your suggestion. We have realized that the evidence in this manuscript will be more convincing if we can prove the directing binding between STAT1 and the promoter region of STING.

We analyzed the BMM Stat1 ChIP-Seq data deposited in GEO (accession number: **GSE33913** and **GSE38377**) from two independent studies (PMID: **22171011** and **23332752**). We found that a significant STAT1 binding peak in the STING promoter. We have further determined the importance of this STAT1 binding site by luciferase reporter assay. Interesting, we also noticed the different intensity of STAT1 binding in this region during IFNβ and IFNγ treatment, and the dynamic change of the STAT1 binding in this region in BMMs treated with IFNγ for different time points. These data are shown in **Fig 2C** and **Fig S2A** of our revised manuscript. Considering ChIP-

Seq data suggest the binding between STAT1 and STING promoter *in vivo*, therefore, we finally decide to show the ChIP-Seq data which provided by other groups and analyzed by us instead of performing EMSA assay. Furthermore, we have verified the ChIP-Seq data by ChIP-qPCR assay (**Fig 2D** of our revised manuscript), IFNβ and IFNγ treatment significant increase the binding between STAT1 and the promoter region of STING. Hopefully, we have made our conclusion more convincing by providing the results from ChIP-Seq, ChIP-qPCR, and luciferase reporter assays.

(4) In Fig.2C, the authors should examine effect of STAT1 expression on STING promoter-dependent expression of luciferase.
Response: Thanks for your suggestions. As data shown in **Fig 2H** of our revised manuscript, overexpression of STAT1 itself in HEK293T is not sufficient to activate the STING promoter reporters significantly, though modest induction of WT-luc and Δ#2-luc reporter was observed after overexpression of STAT1. Probably the signaling that phosphorylates STAT1 is required for inducing its target genes. IFNα treatment or STAT1 transfection plus IFNα treatment significantly activates WT-luc and Δ#2-luc reporters, but not Δ#2-mut#1-luc reporter, which suggests that STAT1#1 is critical for the induction of STING by IFN-I.

(5) In Fig.3, the authors should show level of IRF7, an IFN-inducible gene, and examine whether IRF7 is involved in STING-mediated expression of IFN-beta or not.
Response: Thanks for your suggestions. As you mentioned, we have found that IRF7 is IFNα-inducible (**Fig 1A** of our revised manuscript) and induction of IRF7 by cGAMP is IRF3-depenent (**Fig 4A** of our revised manuscript). We have also tested whether the model proposed in this study is overlapping with the classical IFN positive feedback loop involving IRF7 induction. Given that this is a very important question, we put all these data in a separate figure (**Fig 4** of our revised manuscript) to make this part more detail and comprehensive.

According to our results from *Irf3-/-* and *Irf7-/-* J2-BMMs, we found that induction of *Ifnb* mRNA by cGAMP was IRF3-dependent rather than IRF7-dependent at the early stage (4h post transfection), while induction of *Ifna4* mRNA by cGAMP required both IRF3 and IRF7 at the early stage. At the later stage (16h post cGAMP transfection), both IFNβ and IFNα production were impaired in *Irf7-/-* J2-BMMs as well as in *Irf3-/-* J2-BMMs, though it seemed that IRF3 was more critical for IFN-I induction at this stage. Induction of STING mRNA was defect in *Irf3-/-* at both time points we checked while IRF7 only affected the STING mRNA induction at the later stage. It is well described previously that induction of IFNα genes requires new synthesis of IFN-inducible transcription factor IRF7 during viral infections, and thus IRF7 mediates the type I IFN positive feedback mainly by driving IFNα genes transcription (PMID: **9822609** and **11070172**). Together, we think that IRF3-dependent IFNβ production mediates the induction of STING at the early stage, while IRF7-dependent IFNα production play a role in induction of STING at the later stage. Our data here suggest that the first wave of IFN-I (IFNβ and little amount IFNα4) induces STING and IRF7, induction of STING is directly used to sense more CDNs and amplify the type I IFN production, while new synthetic IRF7 activates IFNα production to initiate the classic type I IFN positive feedback loop by inducing IRF7 itself and STING expression (**Fig 5D** of our revised manuscript).

(6) In Fig.4B, the authors should examine production of IFN-beta by unstimuated J2 BMM.
Response: Thanks for your suggestions. We have added the data for production of IFNβ by unstimuated J2-BMMs (**Fig 5B** of our revised manuscript). Overexpression of STING in unstimulated J2-BMMs triggers production of IFNβ, though there is no difference between WT and *Ifnar1-/-* cells. Transfection of cGAMP induces more IFNβ in WT cells than *Ifnar1-/-* cells, which suggest that induction of STING plays a role in the positive feedback of type I IFN production.

(7) In Fig.4, the authors should compare level of STING between WT BMM stimulated with cGAMP and J2-BMM transduced with LV-STING. If level of STING in J2-BMM is much higher than that in WT BMM, the authors cannot conclude that induction of STING plays a role in IFN-mediated positive feedback loop of cGAS-STING signaling axis.
Response: Thanks for your comments and suggestions. We have examined the STING protein level in WT BMM stimulated with cGAMP and J2-BMM transduced with LV-STING. It seemed that BMMs has higher basal level of STING than J2-BMM, however, the STING levels in cGAMP-activated BMMs and LV-STING transduced J2-BMMs are similar (**Fig S4A** of our revised manuscript).

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Response to Reviewer #2:

In this work Ma et al propose that STING in an IFN-stimulated gene and that this contributes to stimulate positive feed-back for IFN induction. The issue of positive feed-back loops in regulation of IFN expression is well established, most notably through the classical work by Marie and Levy (EMBO J. 1998 Nov 16;17:6660-9.), and the implications of this phenomenon for in vivo biology have been demonstrated several times. The work presented is generally of high quality and the conclusions drawn are supported by the data. Despite this, does this reviewer find that the authors do not provide convincing evidence on the importance of STING induction in stimulation of IFN expression during infection? Even more importantly, the authors make no attempts to relate the proposed positive feed-back loop to the well-established IRF3->IFNb->IRF5->IFNa/b loop.

Response: Thanks for your comments. To address your concerns, we have added new data (**Fig 1A, 1C, 1D, 1H, 1I, 2H, 3B, 3D, 3F, 3H, 3J, 4A-F, 5D, and Supplementary Fig S4B-D**) and discussed in our revised manuscript (the changes have been yellow highlighted). We have also provided point to point responses to your comments and questions as below.

(1) Figure 1. Data on mRNA levels of IRF3 and IRF7 in WT cells have to be provided.

Response: Thanks for your suggestion. We have added mRNA levels of IRF3 and IRF7 from Mock and IFNα-treated WT BMMs (**Fig 1A** of our revised manuscript). As we expected, IRF3 expression is not affected by IFNα treatment, while IRF7 could be induced by IFNα dramatically in BMMs, which is consistent with previous studies showing that IRF7 is an IFN-inducible transcription factor and mediates type I IFN positive feedback loop (PMID: **9877175**, **9822609,** and **11070172**).

(2) Figure 1. STING mRNA induction in IRF7 KO cells should be tested to evaluate whether the proposed mechanism is overlapping with the classical IFN positive feed-back look involving IRF7 induction.

Response: Thanks for your comments and suggestions. We have examined the STING mRNA induction in cGAMP-activated *Irf7-/-* J2-BMMs (**Fig 4A** of our revised manuscript) and tested whether the model proposed by this study is overlapping with the classical IFN positive feedback loop involving IRF7 induction. Given that this is a very important question, we put all these data in a separate figure (**Fig 4** of our revised manuscript) to make this part more detail and comprehensive.

We have found that IRF7 is IFNα-inducible (**Fig 1A** of our revised manuscript) and induction of IRF7 by cGAMP is IRF3-depenent (**Fig 4A** of our revised manuscript). According to our results from *Irf3-/-* and *Irf7-/-* J2-BMMs, we found that induction of *Ifnb* mRNA by cGAMP was IRF3-dependent rather than IRF7-dependent at the early stage (4h post transfection), while induction of *Ifna4* mRNA by cGAMP required both IRF3 and IRF7 at the early stage. At later stage (16h post cGAMP transfection), both IFNβ and IFNα production were impaired in *Irf7-/-* J2-BMMs as well as in *Irf3-/-* J2-BMMs, though it seemed that IRF3 was more critical for cGAMP-triggered IFNβ production at this stage. Induction of STING mRNA was defect in *Irf3-/-* J2-BMMs at both time points we checked, while IRF7 only affected the STING mRNA induction at the later stage. It is well established that induction of IFNα genes requires new synthesis of IFN-inducible transcription factor IRF7 during viral infections, and thus IRF7 mediates the type I IFN positive feedback mainly by driving IFNα genes transcription. Together, we think that IRF3-dependent IFNβ production mediates the induction of STING at the early stage, while IRF7-dependent IFNα production plays a role in induction of STING at the later stage. Our data here suggest that the first wave of IFN-I (IFNβ and little amount IFNα4) induces STING and IRF7, induction of STING is directly used to sense more CDNs and amplify the type I IFN production, while new synthetic IRF7 activates IFNα production to initiate the classic type I IFN positive feedback loop by inducing IRF7 itself and STING expression (**Fig 5D** of our revised manuscript).

(3) Figure 1. The data would gain significantly if data were included from primary human cells and/or on IFNAR-dependent STING induction after infection with a relevant pathogen.

Response: Thanks for your suggestions. We examined the STING mRNA induction by IFNα in human PBMC-derived macrophage and dendritic cells, and found that STING is also an IFN-inducible gene in the human immune cells (**Fig 1C and D** of our revised manuscript).

We have tried cGAMP-related pathogen HSV-1 and c-di-GMP-related pathogen LM. Although we have seen more STING mRNA in WT BMMs than *Ifnar1-/-* BMMs infected with these pathogens, however, we have not observed that significant induction of STING in WT BMMs after infections. As we described in **Fig S1B-C** and **Question 4**, regulation of STING is much more

complicated than "STING is an ISG", HSV-1 and LM does not only activate type I IFN, but also trigger other signaling such as NF-κB and MAPK pathways. To make this study less confusing and highlighting the point "STING is an ISG", we did not include those data. However, we agree with you that this study would gain physiological significance if we include data on IFNAR-dependent STING induction after infection with a relevant pathogen.

(4) Figure 1F. It is surprising that the authors have not included data from untreated cells. It is well established that IFNAR-/- cells have a lower basal expression of ISGs. Therefore, it is central that data from untreated WT and IFNAR -/- cells are included.
Response: Thanks for your comment and suggestion. We repeated this experiment and added data of the time point "0" in our new figure. As shown in **Fig 1H** of our revised manuscript, 1) protein level of STING is comparable between inactivated WT and *Ifnar1-/-* BMMs (time point "0"); 2) STING is induced 12h and 24h by cGAMP post transfection in WT BMMs; 3) Transfection of cGAMP downregulates STING expression in *Ifnar1-/-* BMMs by an unknown mechanism, which is an opposite phenotype in WT BMMs; 4) Significant higher STING level in cGAMP-activated WT than *Ifnar1-/-* BMMs.

As we described in this study, STING is an ISG, therefore it is reasonable that cGAMP could induce STING expression by triggering IFN-I production in WT BMMs. Given that STING could not be induced by cGAMP in *Ifnar1-/-* BMMs, it makes sense that higher STING level was observed in cGAMP-activated WT BMMs than *Ifnar1-/-* BMMs. Interestingly, STING prtoein expression is downregulated by transfection of cGAMP in *Ifnar1-/-* BMMs. We don't know how STING is regulated by cGAMP in *Ifnar1-/-* BMMs by now.

It is well established that *Ifnar1-/-* cells have a lower basal expression of ISGs such as Mx1, Oas2, and IRF7. However, we did not see a lower basal expression of STING in *Ifnar1-/-* BMMs, neither protein level (**Fig 1H** of our revised manuscript) nor mRNA level (**Fig 1E** and **1G** of our revised manuscript). The results from *Ifnar1-/-* BMMs suggest that regulation of STING is much more complicated than "STING is an ISG". Indeed, cGAMP destabilizes STING protein by triggering ULK1 phosphorylation in primary MEF and hTERT-BJ1 cells (PMID: **24119841**), which indicate that cGAMP may play different roles in regulating STING expression. Probably in WT BMMs, activation of BMM by cGAMP triggers both positive feedback via type I IFN production and negative feedback through other pathways such as AMPK-ULK1 pathway. Further studies are required for fully understanding how STING is fine regulated at multiple levels during innate immune responses.

(5) Figure 2C. Can the authors demonstrate that IFN treatment of HEK293T cells activates the reporter gene in a manner dependent on the STAT binding site?
Response: As the new data shown in **Fig 2H** of our revised manuscript, overexpression of STAT1 itself in HEK293T is not sufficient to activate the STING promoter reporters significantly, though modest induction of WT-luc and Δ#2-luc reporter was observed after overexpression of STAT1. Probably the signaling that phosphorylates STAT1 is required for inducing its target genes. IFNα treatment or STAT1 transfection plus IFNα treatment significantly activates WT-luc and Δ#2-luc reporters, but not Δ#2-mut#1-luc reporter, which suggests that STAT1#1 is critical for the induction of STING by IFN-I.

(6)Figure 4A and B. IRF7-/- cells should be transduced with LV-STING and IFNb levels should be determined in order to examine the role of the classical IRF3-IFNb-IRF7-IFNa/b pathway in the proposed pathway.
Response: Thanks for your suggestions, *Irf7-/-* J2-BMMs have been transduced with LV-STING and IFNβ level has been determined in order to examine the role of the classical IRF3-IFNβ-IRF7-IFNα/β pathway in the proposed pathway. As you see in **Supplementary Fig S4B-D**, IRF7 is required for optimal production of cGAMP-triggered IFNβ. Overexpression of STING rescues the impaired production of cGAMP-triggered IFNβ in *Irf7-/-* macrophages. These data suggest that classical IRF3-IFNβ-IRF7-IFNα/β pathway play a role in the proposed pathway by this study. As we described in **Question 2**, IRF7 is required for induction of STING at the later stage of cGAMP transfection, which accounts for how IRF7 and STING work together to play roles in type I IFN positive feedback loop (**Fig 5D** of our revised manuscript).

(7) The model in Figure 4D, should also include classical positive feedback look described by Marie and Levy (EMBO J. 1998 Nov 16;17:6660-9.).

Response: Thanks for your suggestions. We have included classical positive feedback look described by Marie and Levy (EMBO J. 1998 Nov 16;17:6660-9.) in **Fig 5D** of our revised manuscript. As we described in **Question 2**, our study suggests that first wave of IFN-I (IFNβ and little amount IFNα4) induces STING and IRF7, induction of STING is directly used to sense more CDNs and amplify the type I IFN production, while new synthetic IRF7 activates IFNα production to initiate the classic type I IFN positive feedback loop by inducing IRF7 itself and STING expression.

(8)All figures. The authors focus their measurements in IFNb. It would be relevant also to include IFNa-species known to be involved in the later stages of the positive feed-back response.
Response: Thanks for your suggestion. We have checked IFNα4 mRNA by qPCR and total IFNα by ELISA, these data have been shown together with IFNβ mRNA and protein data (**Fig 3B, 3D, 3F, 3H, 3J, 4C, 4E and Supplementary Fig S4D**).

MINOR POINTS
(1) I did not manage to find the data on LPS-stimulated IFN induction (described together with Figure 1G). Should be included.
Response: We have added the data on LPS-stimulated IFN induction in **Fig 1I** of our revised manuscript.

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Response to Reviewer #3:
Overall Comments:
• There are no significant flaws in experimental design or in interpretation of results; findings are interesting though not at all surprising.
• However, the authors do not delineate the novel findings from those that have already been shown by other workers. They do not effectively emphasize the novelty their work (i.e. 1st to show STING is regulated transcriptionally).
• The manuscript is very poorly written.
Response: Thank you very much for your carefully reading. We appreciate your comments and suggestions. With the help from the other reviewers, we have added new data and discussed more in our revised manuscript. To address your concerns, we discussed our novel findings after describing our experimental results in "Results and Discussion" part of the manuscript. In addition, we further discussed: 1) the novelty of our work; 2) the contributions of this study to the field; 3) and the questions need to be further investigated at the end of the "Results and Discussion" part. We have also provided point to point responses to your comments and questions as below. As a result, the manuscript is indeed significantly improved. Hopefully, the manuscript written is also improved. Please let us know if you still have any other questions about this manuscript.

Specific Comments:
Abstract Section
(1) 1st line of abstract section- sentence reads "Stimulator of interferon genes (STING) plays an important role in innate immune response to pathogenic cytoplasmic DNA"...should read "Stimulator of interferon genes (STING) plays an important role in the innate immune response to pathogenic cytoplasmic DNA".
Response: The phrase "…in innate immune response…" has been replaced by "…in **the** innate immune response…" in this sentence.

(2) Last line in the abstract- "Thus, our study has demonstrated that STING is an interferon-stimulated gene (ISG) and its induction plays a role in IFN-I positive feedback loop." Should read..."Thus, our study has demonstrated that STING is an interferon-stimulated gene (ISG) and its induction plays a role in the IFN-I positive feedback loop."
Response: The phrase "…in IFN-I positive feedback loop…" has been replaced by "…in **the** IFN-I positive feedback loop…" in this sentence.

Introduction Section
(1) Last line of the 1st paragraph- sentence reads "Although STING-dependent IFN-I pathway has been extensively studied, it is largely unknown how to regulate STING itself, particularly at the transcriptional level."...should read..."Although the STING-dependent IFN-I pathway has been

extensively studied, it is largely unknown how STING is regulated, particularly at the transcriptional level."
Response: The phrase "…how to regulate STING itself…" has been changed into "…how STING is regulated…"

(2) 3rd sentence in the second paragraph- sentence reads "For example, induction of cytosolic RNA and DNA sensors such as RIG-I, MDA5, and IFI16 enhances the induction of IFN-I by sensing more pathogen nucleic acids." ...Sentence should read..."For example, induction of cytosolic RNA and DNA sensors such as RIG-I, MDA5, and IFI16 enhances the induction of IFN-I by sensing more pathogen-derived nucleic acids."
Response: The phrase "…pathogen nucleic acids…" has been changed into "…pathogen-derived nucleic acids…" in this sentence.

(3)1st line of the last paragraph of the introduction- line reads "Here, our data indicate that STING could be induced by IFN-I via a STAT1 binding site in its promoter region; positive feedback regulation loop is required for optimal production of CDNs-triggered IFN-I." should read..."Here, our data indicate that STING expression could be induced by IFN-I via a STAT1 binding site in its promoter region; positive feedback regulation loop is required for optimal production of CDNs-triggered IFN-I."
Response: The phrase "…STING could be induced…" has been changed into "…STING expression could be induced…" in this sentence.

Results and Discussion Sections
(1)2nd sentence in the first paragraph- sentence reads "However, other genes in the downstream of STING signaling pathway..." sentence should read "However, other genes downstream of the STING signaling pathway..."
Response: The phrase "…the other genes…" has been replaced by "…other genes…" in this sentence.

(2)Rationale and experiment for fig 1C...Is it not well established that STING is an ISG?
Response: Thanks for your suggestion. We have described polyI:C and polydA:dT (the mimics of pathogen-derived nucleic acid which trigger large amount of IFN-I production in immune cells) in our revised manuscript. We think the readers could understand the rationale of this experiment and why we design it with these information. To our knowledge, no study has reported that STING is an ISG. Actually, we think it is our biggest novelty to claim that STING is an ISG.

(3) I would have liked to have seen controls where STING expression was inhibited by shRNAs or through genetic deletion.
Response: Thank you for your comments. Previous studies with STING knockout cells and mice have already demonstrated the critical role of STING in Type I interferon response to viral and bacterial infections (**Reference 1~8** our revised manuscript). The focus of our current studies is to show that STING itself is an ISG. Although we did not use STING knockout or knockdown cells as controls, we do have other controls to makes sure our qPCR and western analyses for STING are specific.

(4)3rd line at the end of the 1st paragraph- sentence reads "Taken together, we have demonstrated that STING transcripts could be induced by IFN-1 and most of the ligands which could trigger IFN-1 in BMMs, therefore demonstrating that STING is an ISG." ...sentence should read "Taken together, we have demonstrated that STING transcripts could be induced by IFN-1 and most of the ligands which could trigger IFN-1 in BMMs, therefore demonstrating that STING is an ISG." Again, I do not think this is a novel claim.
Response: The phrase "…that is to say, STING is an ISG…" has been changed into "…therefore demonstrating that STING is an ISG…" in this sentence. We think it is our biggest contribution of this study to the innate immunity field if we can claim "STING is ISG". ISG is a well-known term for IFN inducible genes, while STING has been recognize as a very important DNA sensor in the past 5 years. It is very interesting and attractive if we can link STING and ISG.

(5)3rd paragraph- sentence reads "Among all predicted TF binding sites around Sting transcription start site (TSS)..." sentence should read "Among all predicted TF binding sites around the Sting transcription start site (TSS)..."

Response: The phrase "…around *Sting* transcription start site (TSS)…" has been changed into "…around **the** *Sting* transcription start site (TSS)…" in this sentence.

(6)4th paragraph- sentence reads "Both sites got high matrix score in the TRANSFAC software..." sentence should read "Both sites got high matrix scores in the TRANSFAC software..."
Response: The phrase "…both of the Stat1 binding sites…" has been changed into "…both sites…" in this sentence.

(7)7th paragraph- sentence reads "...we hypothesized that the induction of STING could increase the host ability of sensing CDNs, and positively regulates CDN-triggered IFN-I production." Sentence should read "...we hypothesized that the induction of STING could increase the host's ability of sensing CDNs, and positively regulates CDN-triggered IFN-I production."
Response: The phrase "…the host ability…" has been changed into "…the host's ability…" in this sentence.

(8) The "discussion" is non-existent!! What do you think the novel mechanism of post-transcriptional regulation of STING-dependent type I interferon production is uniquely contributing to the host innate immune response??? Any speculations?? Probably extremely important to regulate the production of type I interferon during the host response because the over production can lead to severe inflammation and tissue damage. Because of this having multiple regulatory mechanisms is key!
Response: Thanks for your comments and suggestions. In "Results and Discussion" part of the manuscript, we discussed our findings at the end of description of our experimental results. In addition, we further discussed: 1) the novelty of our work; 2) the contributions of this study to the field; 3) and the questions need to be further investigated at the end of the "Results and Discussion" part. We think we have also discussed the points you suggested above. Thanks for your help.

The figures and figure legends
• Figure 1- the letters of each figure need to be aligned and graphs as well ...sloppy
• Figure 2- same comment from figure
Response: Thank you for your comment and suggestion. We have revised Figure 1 and Figure 2, all the letters and graphs of each figure have been aligned. Hope these figures are suitable to be published in *EMBO reports* now.

2nd Editorial Decision 08 December 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports, pending a standard in-house figure check.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

REFEREE REPORTS:

Referee #1:
The manuscript has been considerably improved. The authors have addressed my concerns.

Referee #2:
The authors have dealt with the points raised in a satisfactory manner.

