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# HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I

Su Jin Choi, Hyun-Cheol Lee, Jae-Hoon Kim, Song Yi Park, Tae-Hwan Kim, Woon-Kyu Lee, Duk-Jae Jang, Ji-Eun Yoon, Young-Il Choi, Seihwan Kim, Chul-Joong Kim, Tso-Pang Yao, Jae Jung, Joo-Yong Lee and Jong-Soo Lee

*Corresponding author: Jong-Soo Lee, Chungnam National University*

**Review timeline:**

| | |
|---|---|
| Submission date: | 17 July 2015 |
| Editorial Decision: | 20 August 2015 |
| Revision received: | 16 November 2015 |
| Accepted: | 08 December 2015 |

Editor: David del Alamo

## Transaction Report:

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

1st Editorial Decision 20 August 2015

Thank you for the submission of your manuscript entitled "HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I" and for your patience during the review process. We have now received the reports from the referees, which I copy below.

As you can see from their comments, all three referees are rather supportive of your study, with referee #3 being less enthusiastic but still positive, but point out to a number of significant concerns that will require your attention before your manuscript can be published in The EMBO Journal. I will not repeat here the referee concerns, which in many cases refer to clarifications and further discussion but may require additional experimental evidence. I believe are rather straightforward, but in any case, please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

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REFEREE REPORTS
Referee #1:

The manuscript by Choi et al. identifies HDAC6 as regulator of viral RNA sensing through deacetylation of RIG-1. The authors show that in HDAC6 knockout mice or cell lines where HDAC6 is knocked down, the antiviral response is severely compromised. They further show that RIG-1, a key sensor of RNA viruses, is a target of HDAC6. By using a series of approaches including acetylation mutants of RIG-1 (K909Q/R), the authors further show that HDAC6-mediated deacetylation of RIG-1 is required for RIG-1 activation and induction of the antiviral response.

In general this is a well-presented and interesting study and the data are clear and convincing.
A key issue that has to be resolved is the correlation between the kinetics on the deacetylation of RIG-1 and the antiviral response. The data suggest that RIG-1 binding to and deacetylation by HDAC6 is transient and after 4hrs the response is restored to basal levels. This is supported by the data in Fig1E, F, where the production of IFN-b, IL-6 is transient with similar kinetics. However these findings are contradicted in Fig. 2E and 3C/F where both IFN-b and IL-6 production is constantly lower in the HDAC6-/- cells. This is also relevant for Figure 5C where the timing for the observed co-localisation between RIG-1 and HDAC6 is not shown.

Another key point is the use of the RIG-1 mutants. The K909R mutant, which in principle has lost HDAC6 regulation behaves almost identically to the wild type protein, even if it is predicted to be super-active. This may be due to the overexpression levels used in these experiments. Have the authors checked the relative expression over the endogenous?

The authors should also consider/discuss the possibility that their observations may be at least partly due to the role of HDAC6 in transcriptional regulation.

Other points:

Fig1D,E,F: The type of infection should be indicated in the graphs.

Fig2F: The changes in the levels of pIkBa are minimal suggesting that NF-kB function is not affected, consistent with the studies by Nusinzon and Horvath, 2006?

Fig. 4A: The experiment should be performed in HDAC-/- cells as in this figure the HDAC6-CDM has a significant effect on viral production. In this experiment the data regarding the importance of the catalytic activity of HDAC6 are more convincing for PR8 infection compared to VSV, whereas the catalytic dead mutant has still a significant effect.

Fig.6A, B: Here we see RIG-1 constructs and not HDAC6 as indicated?

Fig 6C: The results for the K858Q/R RIG-1 mutant are confusing. Why there is no stimulation of IFN-b-luc activity since the mutants behave as the wild type protein in terms of sensing dsRNA?

Fig6 D: knockdown of HDAC6 in the RIG-1 KO cells may indicate if the HDAC6-RIG-1 regulation is the key function for HDAC6 in the antiviral response. This will also help to assess the role of RIG-1 independent functions of HDAC6 in the antiviral response.

Referee #2:

In their study "HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I" Choi et al. identified a lysine (K909) in the antiviral cytosolic nucleic acid receptor RIG-I to be involved in a immunoregulatory mechanism. They start with the observation that HDAC6 (deacetylase) deficient mice are more susceptible to VSV-Indiana infection. They found an increased viral load and reduced IFN-beta levels. Additionally, they observed reduced IFN-beta after stimulation of murine BMDM and PBMC with VSV, poly(I:C) and ppp-dsRNA but not dsDNA (dAdT) or dsDNA virus (HSV) pointing to the cytosolic RIG-I like receptor (RLR) pathway as target of HDAC6. Accordingly, they detected less activated IRF-3 and TBK1 after infection with Influenza virus (PR8). Similar results were obtained in the murine RAW (macrophage) cell line treated with siRNAs targeting HDAC6 or in HDAC6 deficient MEFs. Reconstitution of HDAC6 deficient MEFs with overexpressed HDAC6 mostly re-established the WT phenotype while overexpression of an enzymatically inactive HDAC6 failed to do so. HDAC6 deficiency did not influence IFN-beta-Luciferase activity induced by overexpression of MDA5, RIG-I and MAVS (without ligand co-transfection). It was observed that HDAC6 interacted transiently with RIG-I when poly(I:C) was present. Interestingly, RIG-I from HDAC6 deficient cells showed a markedly reduced affinity to ppp-dsRNA, which was reconstituted when HDAC6 was overexpressed. Applying K->Q mutation as equivalent of acetylation, the authors found that one of two previously identified acetylated sites of RIG-I (K858 and K909), K909, mediates loss of ppp-dsRNA binding, if mutated to Q. Remarkably, the acetylation resistant RIG-I

K909R mutant completely re-established the RIG-I response to poly(I:C) as well as the antiviral response to VSV. In pull-down assays with ppp-dsRNA K909R could partially restore binding in HDAC6 k.o., while binding of RIG-I WT and K909R were equal in HDAC6 +/+ MEFs. Applying a newly generated antibody against RIG-I-K909-acetyl, the authors found that HDAC6 deacetylates K909. VSV infection studies reveal a maximum of deacetylation of RIG-I 2h after infection (consistent with HDAC6 interaction). A 3D structure model suggest that acetylation of K909 impairs the essential interaction oft the neighboring K907 with the RNA backbone.

This is an elegant and comprehensive study, demonstrating the mechanism and in vivo relevance of a so far unknown regulation mechanism of an innate immune receptor. It connects HDAC6 which has been implicated in inflammatory diseases and tumor generation, with RIG-I, a crucial antiviral nucleic acid receptor. Animal infection models show in vivo relevance, while expression of mutated proteins or reconstitution experiments in knock-out cells as well as interaction assays resolve the mechanism on a molecular level.
For publication, minor changes concerning data presentation and interpretation needs to addressed.

Minor comments:

1. Several findings point to an additional RIG-I acetylation independent function of HDAC6 in RIG-I activation (Fig. 5F, 6E). The authors should quantify the blot in Fig. 5F and discuss this issue in context of Fig. 5F and 6E (only partial reconstitution of RIG-I binding).

2. The deacetylation experiment does not exclude that HDAC6 also deacetylated other sites of RIG-I and therefore is no proof of specificity for K909. For this purpose, antibodies for other possible sites would be needed. The authors should avoid the word "specificity".

3. The sentence in the discussion "Deacetylation of K907 by HDAC6 may break the hydrogen bond between the K907 and K909 residues, preventing K907 from binding to 5'ppp-dsRNA." is confusing. Maybe 909 was mixed with K907?

4. Fig 4F and G and Supplementary Fig S6B: The sentence "HDAC6 deficiency did not influence IFN-beta-Luciferase activity induced by of overexpression MDA5, RIG-I and MAVS." is misleading. The authors should mention/discuss that they monitor ligand independent IFN induction here - otherwise this result will confuse the reader. A more appropriate experiment would be to overexpress the CARDs of RIG-I which induce type-I-IFN in a ligand-independent manner.

5. Maybe the 3D-modeling results should be transferred in the results part of the paper.

6. What is the biological role of regulation by K909 acetylation? There are species, which possess an arginine at that position.

7. Fig.1F: "infection" should be "injection"

8. Introduce abbreviations HSV, VSV, PR8 in the text before using it.

Referee #3:

EMBOJ-2015-92586
HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I

In this report, the authors provide evidence that HDAC6 is required to deacetylate RIG-I on K909 to allow binding of dsRNA and activation of downstream signaling. In support of this notion, they show that depletion of HDAC6 impairs IFN production and enhances RNA virus replication, that HDAC6 catalytic activity stimulates dsRNA binding by RIG-I and IFN production, and that RIG-I becomes transiently deacetylated on K909 following virus infection.

There are a number of concerns that need to be addressed.

1. It has been shown previously that HDAC6 augments IFN production and induction of an antiviral state. Therefore, much of the data in this study are confirmatory rather than novel.

2. The present study somewhat contradicts previous reports that showed that HDAC6 activity was required for enhanceosome assembly, specifically through augmenting the role of b-catenin, but did not affect IRF3 function per se. This contradiction needs to be addressed directly and robustly. For instance, could the affect on IRF3 phosphorylation observed in this study be secondary to an indirect effect of reduced IFN signaling through b-catenin, for instance, due to impaired feed-forward regulation?

3. Much of the effects documented in this study are relatively modest. In many experiments, the affect of HDAC6 is only 2-3 fold on IFN induction, which remains still very highly inducible in the absence of HDAC6 activity. This raises issues concerning the physiologic relevance of the findings. Similarly, the degree of RIG-1 deacetylation during infection or its impaired interaction with dsRNA in the absence of HDAC6 are relatively small (~2-fold).

4. It would appear that the RIG-I K909Q mutant is completely inactive, raising the concern that this mutation has disrupted its structure, which may not be a mimic of acetylation. Note that the K909Q mutant appears to have no activity (Fig. 6D), while WT RIG-I retains significant activity, even in the absence of HDAC6. Similarly, RIG-I K909R displays reduced activity in absence of HDAC6, suggesting at a minimum that there are additional targets for HDAC6. Thus, one is left with the question of how important is RIG-I deacetylation to the overall role of HDAC6 in innate immunity.

5. What is the basis for the transient nature of the HDAC6 interaction with RIG-I?

6. Why does RIG-I expression induce IFN transcription (Fig. 6F)? This result is different from many previous reports that shown that RIG-I activity is completely dependent on dsRNA.

1st Revision - authors' response 16 November 2015

**Point-by-Point Response to Referee #1's Comments**

**Comments)** The manuscript by Choi et al. identifies HDAC6 as regulator of viral RNA sensing through deacetylation of RIG-1. The authors show that in HDAC6 knockout mice or cell lines where HDAC6 is knocked down, the antiviral response is severely compromised. They further show that RIG-1, a key sensor of RNA viruses, is a target of HDAC6. By using a series of approaches including acetylation mutants of RIG-1 (K909Q/R), the authors further show that HDAC6-mediated deacetylation of RIG-1 is required for RIG-1 activation and induction of the antiviral response. In general this is a well-presented and interesting study and the data are clear and convincing.

***Response)*** *We thank the referee for encouraging comment and valuable suggestions.*

**Comments)** A key issue that has to be resolved is the correlation between the kinetics on the deacetylation of RIG-1 and the antiviral response. The data suggest that RIG-1 binding to and deacetylation by HDAC6 is transient and after 4hrs the response is restored to basal levels. This is supported by the data in Fig1E, F, where the production of IFN-b, IL-6 is transient with similar kinetics. However these findings are contradicted in Fig. 2E and 3C/F where both IFN-b and IL-6

production is constantly lower in the HDAC6-/- cells. This is also relevant for Figure 5C where the timing for the observed co-localization between RIG-1 and HDAC6 is not shown.

***Response)*** *We thank the reviewer for insightful comments. As referee's comments, Figure 2E and 3C/F could be seen not to have similar kinetics with Figure 1E and F. As described in our manuscript, HDAC6 is involved in deacetylation of RIG-I for the viral RNA recognition in early time of virus infection. In accordance with this interaction, we have shown that secretion of IFN-β or IL-6 were reduced in HDAC6$^{-/-}$ mice infected with VSV-GFP virus at the early time phase (Figure 1E). We also presented the induction of IFN-β or antiviral gene at the early time in vitro (Figure 2G, Appendix Figure S5). Also, after transfection with 5'ppp-dsRNA, we found that RIG-I and HDAC6 were co-localized at 8 hours which is the same time point that we observed the interaction in Figure 5C (We apology for missing information about timing. Now, we included the information in page 11 line 11, 16 and Figure 5C legend). However, in case of Figure 2E and 3C/F, we measured the levels of secreted IFN-β or IL-6 from supernatant of cell lines which infected with viruses or treated with a synthetic ligand for RIG-I at 12h, 24h and it could reflect the total amount of secreted cytokine from the cells. As shown in (Oshiumi et al, 2010; Sun et al, 2006), regulation of upstream molecules in innate immunity could trigger secretion of cytokine levels differentially in supernatant at late time in vitro.*

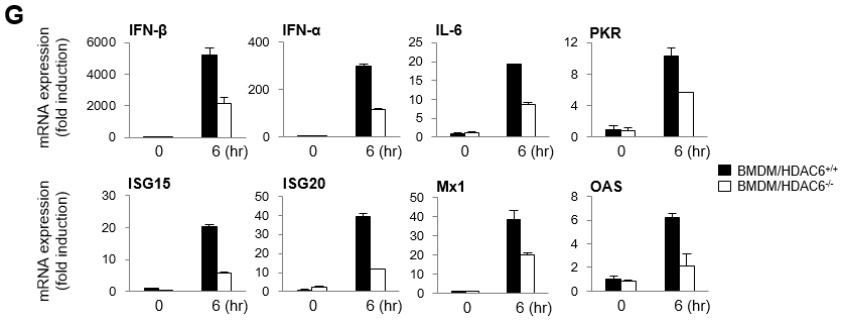



**Figure 2G**

**Supporting references:**

1. Oshiumi H, Miyashita M, Inoue N, Okabe M, Matsumoto M, Seya T (2010) The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. Cell host & microbe 8: 496-509 **(505)**
2. Sun Q, Sun L, Liu HH, Chen X, Seth RB, Forman J, Chen ZJ (2006) The specific and essential role of MAVS in antiviral innate immune responses. Immunity 24: 633-642 **(636-638)**

**Comments)** Another key point is the use of the RIG-1 mutants. The K909R mutant, which in principle has lost HDAC6 regulation behaves almost identically to the wild type protein, even if it is predicted to be super-active. This may be due to the overexpression levels used in these experiments. Have the authors checked the relative expression over the endogenous?

***Response)*** *We thank the reviewer for insightful suggestions. As suggested, we tried to confirm the expression levels between overexpressed RIG-I and endogenous RIG-I. As the expectation of referee, activities of wild type RIG-I and RIG-I K909R mutant might have been shown the similar pattern by the levels of overexpressed RIG-I or RIG-I K909R mutant (Figure 6C).*

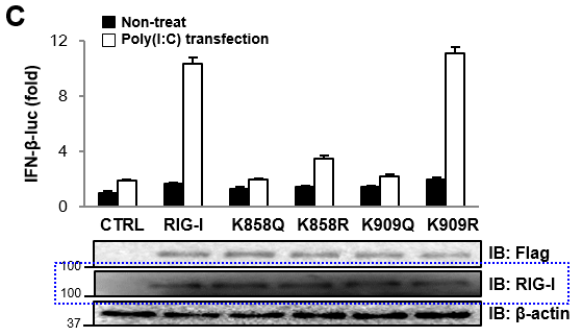
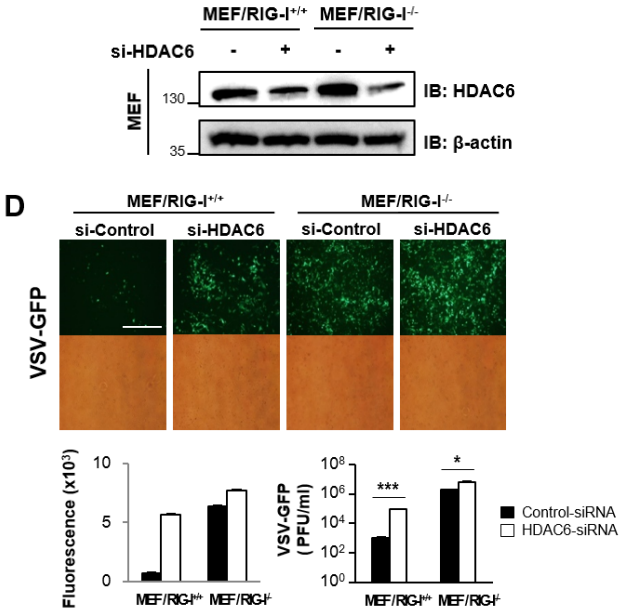



**Figure 6C**

**Comments)** The authors should also consider/discuss the possibility that their observations may be at least partly due to the role of HDAC6 in transcriptional regulation.

***Response)*** *We appreciate for the referee's very useful comments. As suggested, we confirmed the RIG-I independent function of HDAC6 in new Figure 5D and Appendix Figure S9. As shown in figure, we observed the partial effect by RIG-I independent function of HDAC6. These results of HDAC6 could be due to β-catenin dependent signaling (Zhu et al, 2011) rather than transcriptional regulation by histone dependent chromatin remodeling.*




**Appendix Figure S9 and Figure 5D**

*To demonstrate this point, first we showed that β-catenin dependent signaling partially involved in HDAC6 related antiviral immune response in new Figure EV4.*

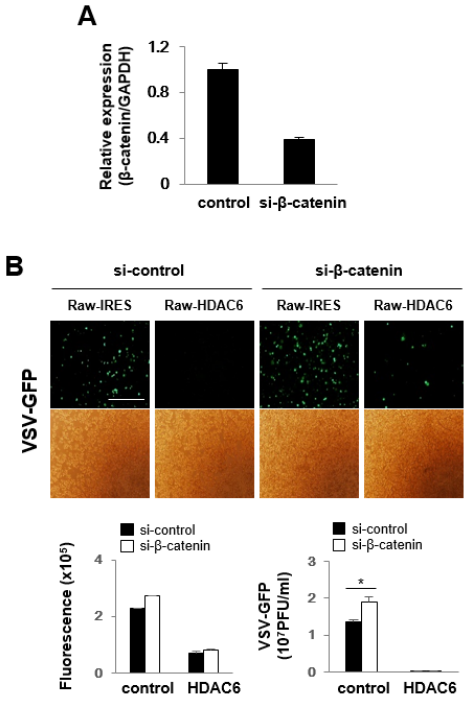
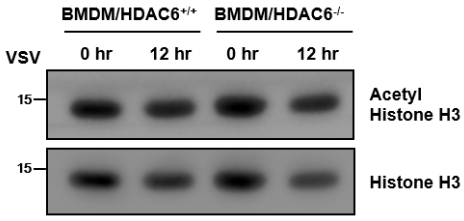



**Figure EV4**

*Second, we showed the localization of HDAC6 is mainly in cytoplasm before and after 5'ppp-dsRNA transfection instead of nuclear (Figure 5C). Additionally, we show that HDAC6 does not affect the level of histone H3 acetylation in HDAC6$^{-/-}$ BMDMs before or after VSV infection (Appendix Figure S12).*




**Appendix Figure S12**

*Taken together, HDAC6 mainly regulates cytoplasmic regulatory machinery to activate antiviral immune responses through RIG-I and also regulates the antiviral immune responses through β-catenin partially.*
*We included this discussion in page 17 line 3-17.*

**Supporting references:**

1. Zhu J, Coyne CB, Sarkar SN (2011) PKC alpha regulates Sendai virus-mediated interferon induction through HDAC6 and beta-catenin. The EMBO journal 30: 4838-4849

**Other points**

**Comments)** Fig1D,E,F: The type of infection should be indicated in the graphs.

***Response)*** *We apology for the mistake and we have added the type of infection in Figure 1D, E, F.*

**Comments)** Fig2F: The changes in the levels of pIkBa are minimal suggesting that NF-kB function is not affected, consistent with the studies by Nusinzon and Horvath, 2006?

***Response)*** *The activation of RIG-I causes phosphorylation of other downstream proteins including IkBα (Loo & Gale, 2011). We have tried additional experiment and now we could show more significant different between HDAC6$^{+/+}$ and HDAC6$^{-/-}$ cells in changed figure. 2F, 5$^{th}$ line.*

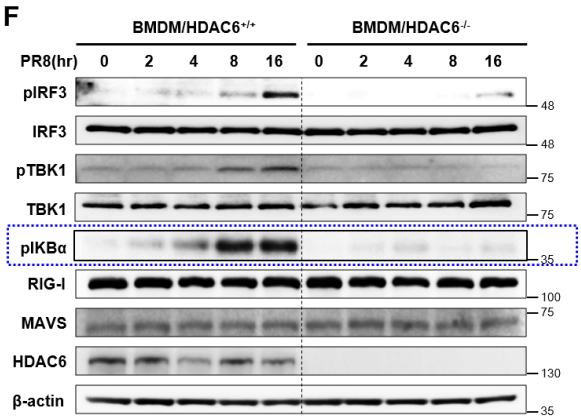



**Figure 2F**

**Supporting references:**

1. Loo YM, Gale M, Jr. (2011) Immune signaling by RIG-I-like receptors. Immunity 34: 680-692 **(684)**

**Comments)** Fig. 4A: The experiment should be performed in HDAC-/- cells as in this figure the HDAC6-CDM has a significant effect on viral production. In this experiment the data regarding the importance of the catalytic activity of HDAC6 are more convincing for PR8 infection compared to VSV, whereas the catalytic dead mutant has still a significant effect.

***Response)*** *As suggested by referee, we performed PR8-GFP infection experiment after transient reconstitution of HDAC6 and HDAC6-CDM in HDAC6$^{-/-}$ MEF cells. As shown in following Figure EV1, we have confirmed catalytic activity of HDAC6 is critical for antiviral effect again, consistent with our Figure 4.*

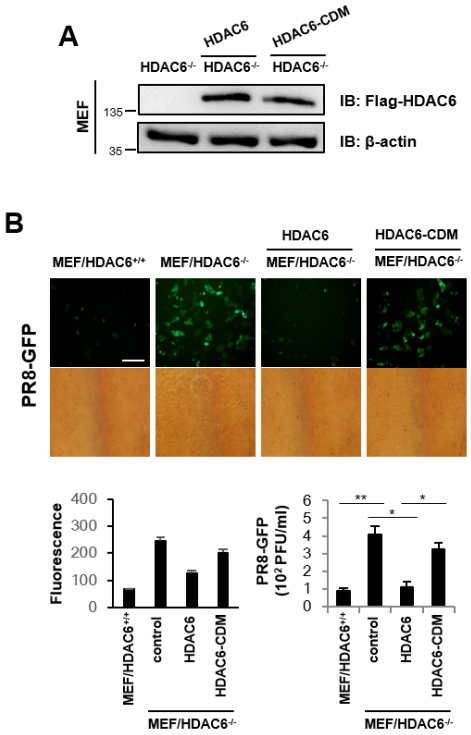



**Figure EV1**

**Comments)** Fig.6A, B: Here we see RIG-1 constructs and not HDAC6 as indicated?

***Response)*** *We are sorry for the mistake and we have corrected the typographical error in Fig. 6A, B*

**Comments)** Fig 6C: The results for the K858Q/R RIG-1 mutant are confusing. Why there is no stimulation of IFN-b-luc activity since the mutants behave as the wild type protein in terms of sensing dsRNA?

***Response)*** *We thank the reviewer for insightful suggestions. As reviewer indicated, we performed the experiment for the induction of IFN-β-luc activity with K858Q/R RIG-1 mutant several times. As shown in figure, we could see the slightly increase level of IFN-β-luc activity by RIG-I K858R mutant only. And also, RIG-I K858Q mutant binds 5'ppp-dsRNA but the affinity is less than wild type RIG-I, indicating K858 are also involved in 5'ppp-dsRNA binding as reported in previous papers (Cui et al, 2008; Lu et al, 2010; Wang et al, 2010). In fact, previous structural study showed that K858 residue directly binds to 5'-triphosphophate of dsRNA. However, RIG-I mutations in K858 residues (K858A, K858E) abolished dsRNA interaction and the RIG-I dependent IFN-β-luc activity (Cui et al, 2008; Lu et al, 2010; Wang et al, 2010) as we observed in Fig 6C. Consequently, this phenotype remains in vague whether K858 site of RIG-I is acetylated and what is the role of its. Now, we corrected result and discussion section (page 19 line 7-13) accordingly.*

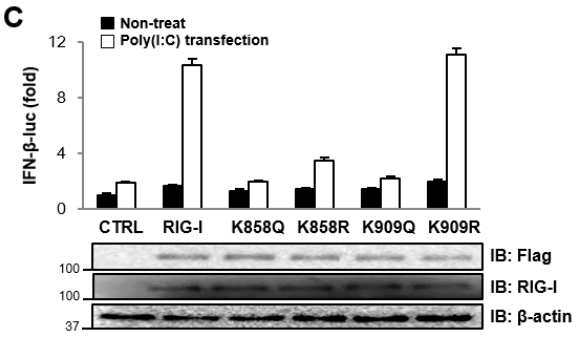


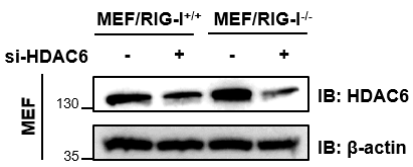

**Figure 6C**

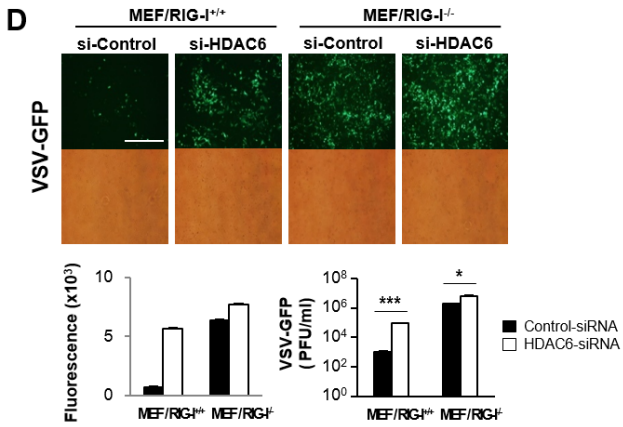
**Supporting references:**

1. Cui S, Eisenacher K, Kirchhofer A, Brzozka K, Lammens A, Lammens K, Fujita T, Conzelmann KK, Krug A, Hopfner KP (2008) The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. Molecular cell 29: 169-179 **(176)**
2. Lu C, Xu H, Ranjith-Kumar CT, Brooks MT, Hou TY, Hu F, Herr AB, Strong RK, Kao CC, Li P (2010) The structural basis of 5' triphosphate double-stranded RNA recognition by RIG-I C-terminal domain. Structure (London, England : 1993) 18: 1032-1043 **(1036-1039)**
3. Wang Y, Ludwig J, Schuberth C, Goldeck M, Schlee M, Li H, Juranek S, Sheng G, Micura R, Tuschl T, Hartmann G, Patel DJ (2010) Structural and functional insights into 5'-ppp RNA pattern recognition by the innate immune receptor RIG-I. Nature structural & molecular biology 17: 781-787 **(783-785)**

**Comments)** Fig6 D: knockdown of HDAC6 in the RIG-1 KO cells may indicate if the HDAC6-RIG-1 regulation is the key function for HDAC6 in the antiviral response. This will also help to assess the role of RIG-1 independent functions of HDAC6 in the antiviral response.

***Response)*** *We thank the referee for insightful comment. As suggested, we tried knock-down of HDAC6 in RIG-I$^{+/+}$ and RIG-I$^{-/-}$ MEF using siRNA, and infected with VSV-GFP to assess this RIG-I independent functions of HDAC6 (Figure 5D and Appendix Figure S9). As shown in below, we found that Knock-down of HDAC6 affects more significant increase of viral replication in RIG-I$^{+/+}$ MEFs (about 70 fold) while RIG-I$^{-/-}$ MEFs showed less changes (about 3 folds). Our new result suggests that antiviral function of HDAC6 is mainly associated with RIG-I even though it has still RIG-I independent functions.*






**Appendix Figure S9 and Figure 5D**

**Point-by-Point Response to Referee #2's Comments**

**Comments)** In their study "HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I" Choi et al. identified a lysine (K909) in the antiviral cytosolic nucleic acid receptor RIG-I to be involved in a immunoregulatory mechanism. They start with the observation that HDAC6 (deacetylase) deficient mice are more susceptible to VSV-Indiana infection. They found an increased viral load and reduced IFN-beta levels. Additionally, they observed reduced IFN-beta after stimulation of murine BMDM and PBMC with VSV, poly(I:C) and ppp-dsRNA but not dsDNA (dAdT) or dsDNA virus (HSV) pointing to the cytosolic RIG-I like receptor (RLR) pathway as target of HDAC6. Accordingly, they detected less activated IRF-3 and TBK1 after infection with Influenza virus (PR8). Similar results were obtained in the murine RAW (macrophage) cell line treated with siRNAs targeting HDAC6 or in HDAC6 deficient MEFs. Reconstitution of HDAC6 deficient MEFs with overexpressed HDAC6 mostly re-established the WT phenotype while overexpression of an enzymatically inactive HDAC6 failed to do so. HDAC6 deficiency did not influence IFN-beta-Luciferase activity induced by overexpression of MDA5, RIG-I and MAVS (without ligand co-transfection). It was observed that HDAC6 interacted transiently with RIG-I when poly(I:C) was present. Interestingly, RIG-I from HDAC6 deficient cells showed a markedly reduced affinity to ppp-dsRNA, which was reconstituted when HDAC6 was overexpressed. Applying K->Q mutation as equivalent of acetylation, the authors found that one of two previously identified acetylated sites of RIG-I (K858 and K909), K909, mediates loss of ppp-dsRNA binding, if mutated to Q. Remarkably, the acetylation resistant RIG-I K909R mutant completely re-established the RIG-I response to poly(I:C) as well as the antiviral response to VSV. In pull-down assays with ppp-dsRNA K909R could partially restore binding in HDAC6 k.o., while binding of RIG-I WT and K909R were equal in HDAC6 +/+ MEFs. Applying a newly generated antibody against RIG-I-K909-acetyl, the authors found that HDAC6 deacetylates K909. VSV infection studies reveal a maximum of deacetylation of RIG-I 2h after infection (consistent with HDAC6 interaction). A 3D structure model suggest that acetylation of K909 impairs the essential interaction of the neighboring K907 with the RNA backbone.

This is an elegant and comprehensive study, demonstrating the mechanism and in vivo relevance of a so far unknown regulation mechanism of an innate immune receptor. It connects HDAC6 which has been implicated in inflammatory diseases and tumor generation, with RIG-I, a crucial antiviral nucleic acid receptor. Animal infection models show in vivo relevance, while expression of mutated proteins or reconstitution experiments in knock-out cells as well as interaction assays resolve the mechanism on a molecular level.
For publication, minor changes concerning data presentation and interpretation needs to addressed.

***Response)*** *We are very thankful to the referee for favorable comments and valuable advices.*

**Minor comments:**
**Comments)** 1. Several findings point to an additional RIG-I acetylation independent function of HDAC6 in RIG-I activation (Fig. 5F, 6E). The authors should quantify the blot in Fig. 5F and discuss this issue in context of Fig. 5F and 6E (only partial reconstitution of RIG-I binding).

***Response)*** *We thank the reviewer for insightful suggestions. As suggested, we quantified the blot in Figure 5F and 5G.*

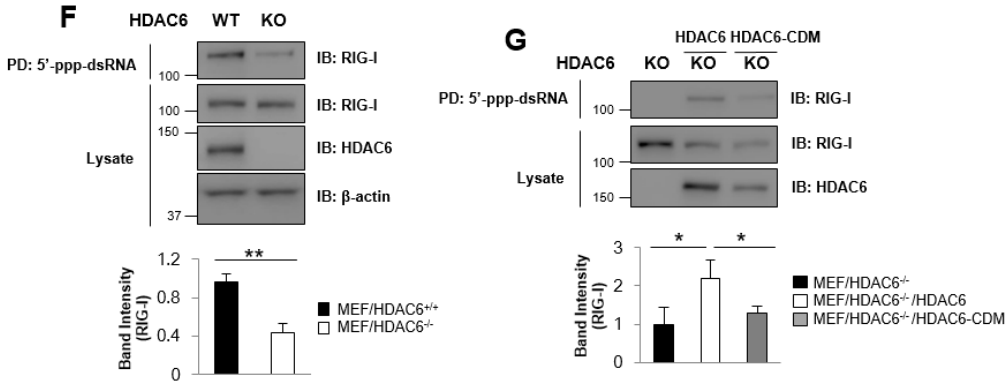
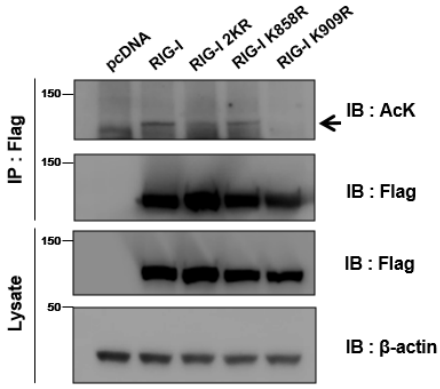



**Figure 5F and 5G**

*To check additional acetylation site of RIG-I, we checked acetylations of RIG-I wild type, K858R, K909R and 2KR (K858R/K909R), using pan-acetyl-lysine antibody. As shown in new Appendix Figure S11, K909R and 2KR mutant of RIG-I showed minimal acetylation, suggesting K909 site is the major site of acetylation. However, we could not completely exclude the possibility of additional acetylation sites due to the specificity of anti-acetyl lysine antibody. Thus, it is still possible that additional acetylation sites of RIG-I involved in the regulation of RIG-I.*
*We followed the reviewer's suggestion and include the discussion about this issue in page 19 line 7-13.*




**Appendix Figure S11**

**Comments)** 2. The deacetylation experiment does not exclude that HDAC6 also deacetylated other sites of RIG-I and therefore is no proof of specificity for K909. For this purpose, antibodies for other possible sites would be needed. The authors should avoid the word "specificity".
***Response)*** *As reviewer's comments, we removed the word "specific" for K909 (also, please see response 1).*

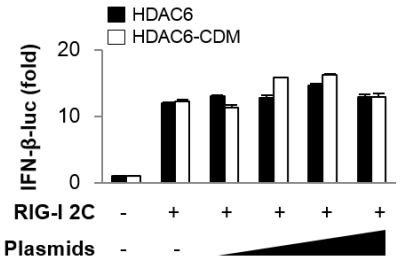
**Comments)** 3. The sentence in the discussion "Deacetylation of K907 by HDAC6 may break the hydrogen bond between the K907 and K909 residues, preventing K907 from binding to 5'ppp-dsRNA." is confusing. Maybe 909 was mixed with K907?
***Response)*** *We apology for the mistake and now it was corrected.*

**Comments)** 4. Fig 4F and G and Supplementary Fig S6B: The sentence "HDAC6 deficiency did not influence IFN-beta-Luciferase activity induced by of overexpression MDA5, RIG-I and MAVS." is misleading. The authors should mention/discuss that they monitor ligand independent IFN induction here - otherwise this result will confuse the reader. A more appropriate experiment would be to overexpress the CARDs of RIG-I which induce type-I-IFN in a ligand-independent manner.

***Response)*** *We thank the reviewer for insightful suggestions. We apologize for the misleading statement. We replaced the sentence in the the manuscript in accordance with the referee's comment. Furthermore, we added IFN-β luciferase result using the 2CARD of RIG-I to properly present ligand-independent manner in Appendix Figure S8A.*

*-Before : Expression of RIG-I, MDA5, and MAVS did not increase the IFN-β luciferase activity when HDAC6 or HDAC6-CDM was co-transfected in a dose-dependent manner (Fig 4F and G and Supplementary Fig S6B).*
*-Replaced : Ligand independent IFN-β induction by RIG-I, MDA-5, MAVS, and 2CARD of RIG-I expression were not increased by co-transfection of HDAC6 or HDAC6-CDM in a dose-dependent manner (Fig 4F and G and Appendix Figure S8A).*




**Appendix Figure S8A**

**Comments)** 5. Maybe the 3D-modeling results should be transferred in the results part of the paper.

***Response)*** *According to reviewer's suggestion, we moved 3D-modeling data to new Figure 7.*

**Comments)** 6. What is the biological role of regulation by K909 acetylation? There are species, which possess an arginine at that position.

***Response)*** *We thank the reviewer for careful suggestions. As shown in our figures, ectopic expressed (Figure 6F and Figure EV3) and endogenous (Figure 6G and H) RIG-I conserves acetylation of 909$^{th}$ lysine, which may prevent the activation of RIG-I. This suggests that RIG-I is maintained to suppress its sensing function by acetylation in uninfected condition. Therefore, we hypothesis that this suppression would be dissolved by HDAC6 which is a deacetylating enzyme, to trigger RIG-I mediated antiviral signaling in response to virus infection.*

*As suggested by referee, we checked the amino acid sequence homology of RIG-I between various species but we could not find arginine in K909 site (new Appendix Figure S10). In fact, K909 site are well preserved from human to C. elegance, but not in Xenopus.*

```
H.Sapiens          835   GDAF-KECFVSRPHPKPKQFSSFEKRAKIFCARQNCSHDWGIHVKYKTF-   882
P.Troglodytes      835   GDAF-KECFVSRPHPKPKQFSSFEKRAKIFCARQNCSHDWGIHVKYKTF-   882
M.mulatta          835   GDAF-KECFVRRPHPKPKKFSNFDKREKIFCARQNCSHDWGIHMKYKTF-   882
C.lupus            833   GDAF-TKCFVSKLHPKPKSFGHFEKRAKIFCARRNCGHDWGIHVKYKTF-   880
B.Taurus           913   RDAF-RECFVTKLHPRPKKFGSFDKKAKIFCARKDCLHDWGIHMKYKTF-   960
M.Musculus         836   GDAF-KERFVCKPHPKPKIYDNFEKKAKIFCAKQNCSHDWGIFVRYKTF-   883
C.Elegans          915   DPGFWSLVRKTRLTDEQQALIKYNATGSINCRRENCGLKLGQLIEVNTV-   963
X.Tropicalis       870   DKTF-KDRYITKKHPKPRTFEGYKKMYKIFCKRPECHEDWGVSGTYQGFQ   918
                                          858

H.Sapiens          883   EIPVIKIESFVVEDIATGVQTLYSKWKDF------HFEKIPFDPAEMSK-   925
P.Troglodytes      883   EIPVIKIESFVVEDIATGVQTLYSKWKDF------HFEKIPFDPAEMSK-   925
M.Mulatta          883   EIPVIKIESFVVEDIATGVQTLYSKWKDF------HFEKIPFDPAEMAK-   925
C.Lupus            881   EIPVIKIESFVVEDIATGAQKLYAKWKDF------PFEKIPFGSPEIPE-   923
B.Taurus           961   EIPVIKIESFVVEDVATGAQTLYAKWKDF------NFEKIPFDAAEMSPW   1004
M.Musculus         884   EIPVIKIESFVVEDIVSGVQNRHSKWKDF------HFERIQFDPAEMSV-   926
C.Elegans          964   DLPCLSALSIVLLVEGTDKRIIVKKWKNILDKYFTPTEIRQLDVQTMRDA   1013
X.Tropicalis       919   DLPLIKIEQFVIEN-PDGTQEYKDKWVDV------HFTMKKLSTEEFLSS   961
                                          909
```

**Appendix Figure S10**

**Comments)** 7. Fig.1F: "infection" should be "injection"

***Response)*** *We corrected the typographical error in the manuscript.*

**Comments)** 8. Introduce abbreviations HSV, VSV, PR8 in the text before using it.
***Response)*** *We introduced the abbreviations to the manuscript.*

**Point-by-Point Response to Referee #3's Comments**

**Comments)** In this report, the authors provide evidence that HDAC6 is required to deacetylate RIG-I on K909 to allow binding of dsRNA and activation of downstream signaling. In support of this notion, they show that depletion of HDAC6 impairs IFN production and enhances RNA virus replication, that HDAC6 catalytic activity stimulates dsRNA binding by RIG-I and IFN production, and that RIG-I becomes transiently deacetylated on K909 following virus infection. There are a number of concerns that need to be addressed.

***Response)*** *We would like to thank the referee for providing insightful comments and useful suggestions. We revised the manuscript in accordance with referee's comments and attempted our best to address the given comments.*

**Comments)** 1. It has been shown previously that HDAC6 augments IFN production and induction of an antiviral state. Therefore, much of the data in this study are confirmatory rather than novel.

***Response)*** *We thank the reviewer for the comment. HDAC6 was firstly reported as a regulator of immune response by Nusinzon & Horvath in 2006. In this paper, they had shown that the broad HDAC inhibitor TSA enhances virus replication and presented the role of HDAC1, 6, 8 using siRNA-mediated knockdown system. However, the paper does not contain the specific molecular mechanism how HDAC6 modulates innate immunity except IFN-β, IRF-3, NF-kB promoter luciferase assay against dsRNA transfection. Although the study suggested the important clue that HDAC6 is involved in innate immunity, the exact mechanism is remaining to be determined. Secondly, Zhu et al reported that PKC alpha regulated the innate immune response and it is mediated by HDAC6-mediated activation of β-catenin, which is responsible for enhancesome formation. In this paper, HDAC6 affords the means of interlink because the main topic of this study was antiviral effect of PKC alpha and its association with enhancesome formation. It is well known that HDAC6 is associated with β-catenin, so that they did not perform the experiment presenting HDAC6 and its direct role in immune response against virus.*

*The first importance of our study is that we revealed regulation of type I interferon by HDAC6 in vivo. We have directly shown virus titrations and ELISA of IFN secretion not only in organs and serum, but also in bone marrow derived macrophages and peripheral blood mononuclear cells, which has not been reported previously. Secondly, for the first time, we demonstrated the relevance of HDAC6 with RIG-I, the crucial molecule in innate immunity. RIG-I is the key molecule that*

*recognize viral genome and undergoes many post translational modification including phosphorylation, ubiquitination, SUMOylation. It had been reported that RIG-I undergoes acetylation, but the mediators and the specific function of this modification had been unknown. We have shown that HDAC6 is deacetylase and regulates this modification at 909$^{th}$ lysine of RIG-I. One of the interesting outcome of our study is that RIG-I could not bind with RNA ligand without deacetylation of HDAC6. It means that the recognition function of RIG-I is tightly controlled by acetylation and HDAC6 is the key molecule to open the recognizing function of RIG-I. It suggests the possibility that the mechanism which, recognized virus infection in host cell could be more complicated than our understating until now.*

**Supporting references:**

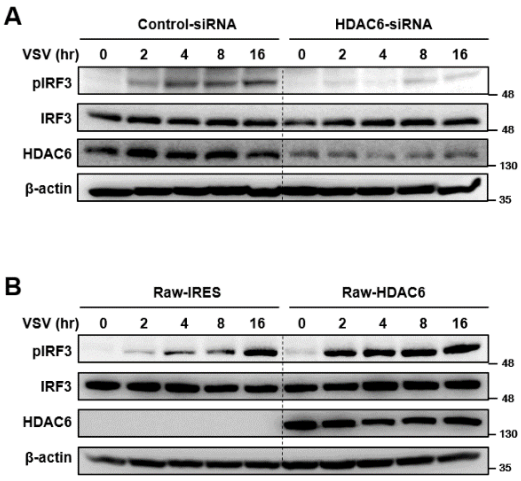
1. Nusinzon I, Horvath CM (2006) Positive and negative regulation of the innate antiviral response and beta interferon gene expression by deacetylation. Molecular and cellular biology 26: 3106-3113
2. Zhu J, Coyne CB, Sarkar SN (2011) PKC alpha regulates Sendai virus-mediated interferon induction through HDAC6 and beta-catenin. The EMBO journal 30: 4838-4849

**Comments)** 2. The present study somewhat contradicts previous reports that showed that HDAC6 activity was required for enhanceosome assembly, specifically through augmenting the role of b-catenin, but did not affect IRF3 function per se. This contradiction needs to be addressed directly and robustly.
For instance, could the affect on IRF3 phosphorylation observed in this study be secondary to an indirect effect of reduced IFN signaling through b-catenin, for instance, due to impaired feed-forward regulation?

***Response)*** *We thank the referee for helpful comment to improve our work. We could find three related paper about HDAC6 and IRF3 activation. First, Nusinzon & Horvath performed luciferase assay using IRF3 promoter. In this paper, they reported that IRF3 promoter assay is affected by siHDAC6 in response to dsRNA stimulation. Second, Zhu et al reported that PKCa induce HDAC6 mediated activation of β-catenin. However, they had considered HDAC6 as an interlink of this mechanism, so that the relation between HDAC6 and IRF3 activation was not assessed although they investigated that PKCα has no effect on IRF activation. Lastly, it was reported by Chattopadhyay et al that HDAC6 knockdown did not affect IRF3 activation in response to TLR3 stimulation. In this study, poly(I:C) was used to stimulate TLR3 in HT1080, human epithelial cell line. Therefore, it might be not enough to turn on the RLR signaling, which is started from RIG-I to induce IRF3 activation.*

*As suggested by referee, we have assessed IRF3 activation in Raw264.7 cell line transfected with siHDAC6 and control (Appendix Figure S3).* ***A)*** *In response to VSV-GFP, phosphorylation of IRF3 reduced in HDAC6 knockdown, which correlates with HDAC6 deficiency in BMDMs.* ***B)*** *Besides,*

*we have investigated IRF3 activation in control and HDAC6 overexpressed RAW264.7 cell line. The HDAC6 overexpressed RAW264.7 cell line used in our work have been proved that HDAC6 induces more activation level of IRF3 than control in the early time of virus infection, suggesting that this variation due to feed-forward by direct regulation of HDAC6 rather than feed-back by secondary indirect effect.*




**Appendix Figure S3**

*Additionally, as the above response for comments of referee 1, we show that β-catenin dependent signaling partially involved in HDAC6 related antiviral immune response in new Figure EV4. Further, based on our studies, HDAC6 mainly regulates cytoplasmic regulatory machinery to activate antiviral immune responses through RIG-I and also regulates the antiviral immune responses through β-catenin partially.*

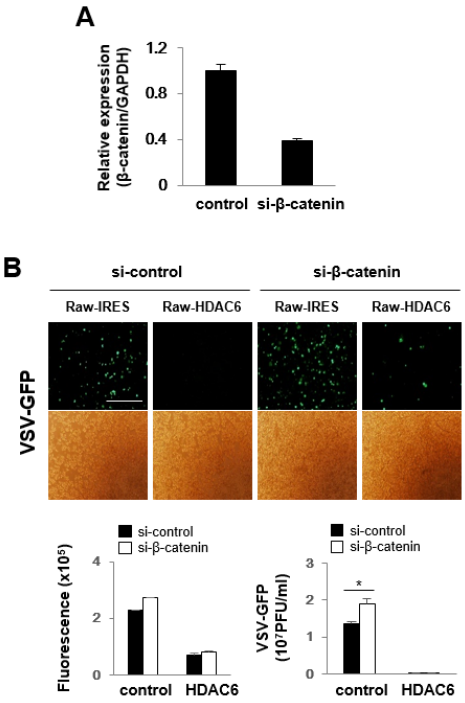



**Figure EV4**

**Supporting references:**


1. Nusinzon I, Horvath CM (2006) Positive and negative regulation of the innate antiviral response and beta interferon gene expression by deacetylation. Molecular and cellular biology 26: 3106-3113 **(3109)**
2. Zhu J, Coyne CB, Sarkar SN (2011) PKC alpha regulates Sendai virus-mediated interferon induction through HDAC6 and beta-catenin. The EMBO journal 30: 4838-4849
3. Chattopadhyay S, Fensterl V, Zhang Y, Veleeparambil M, Wetzel JL, Sen GC (2013) Inhibition of viral pathogenesis and promotion of the septic shock response to bacterial infection by IRF-3 are regulated by the acetylation and phosphorylation of its coactivators. MBio 4: e00636-00612 **(e00636-12)**


**Comments)** 3. Much of the effects documented in this study are relatively modest. In many experiments, the affect of HDAC6 is only 2-3 fold on IFN induction, which remains still very highly inducible in the absence of HDAC6 activity. This raises issues concerning the physiologic relevance of the findings. Similarly, the degree of RIG-1 deacetylation during infection or its impaired interaction with dsRNA in the absence of HDAC6 are relatively small (~2-fold).

***Response)*** *We thank the referee for raising the point. Innate immunity consists of various intracellular signaling and it interacts with each other intricately within complicated network. For RNA virus infection, it has been reported that several molecules are involved in antiviral defense besides RIG-I pathway. Due to this complicated network, effect of single molecule could be induce modest result even it affect RIG-I. Even though, induction could be modest, it should be robust. Thus, our hypothesis is supported by below figures which is assessed in various cells including BMDMs, PBMCs, RAW264.7 and MEFs tested with several stimulants.*

*Additionally, figures about activation of signaling molecules dramatically show effects of HDAC6, which could more accurately reflect regulation of RIG-I. We repeated some of the experiments, and replaced our previous modest results of ELISA (Figure 2E and Figure 3C), immunoblot (Figure 6G and H) and added quantitative analysis of western blots (Figure 6G and H) to resolve the concerns of the reviewer.*

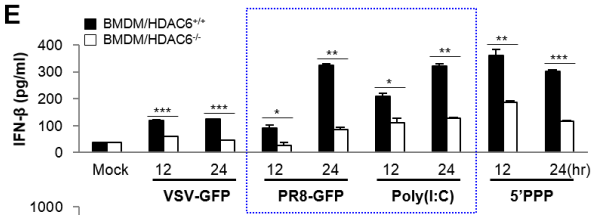



**Figure 2E**

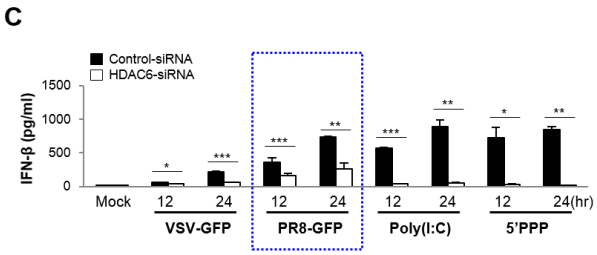



**Figure 3C**

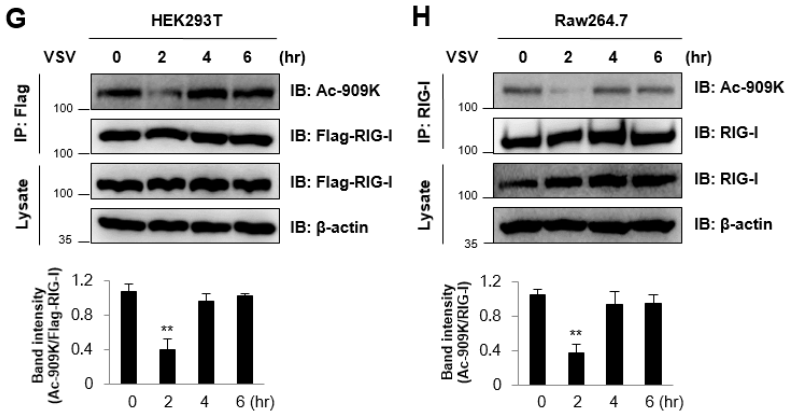



**Figure 6G and 6H**

**Comments)** 4. It would appear that the RIG-I K909Q mutant is completely inactive, raising the concern that this mutation has disrupted its structure, which may not be a mimic of acetylation. Note that the K909Q mutant appears to have no activity (Fig. 6D), while WT RIG-I retains significant activity, even in the absence of HDAC6. Similarly, RIG-I K909R displays reduced activity in absence of HDAC6, suggesting at a minimum that there are additional targets for HDAC6. Thus, one is left with the question of how important is RIG-I deacetylation to the overall role of HDAC6 in innate immunity.

***Response)*** *We thank the reviewer for the comment. The fact that our ac909K-specific antibody was generated using peptides containing acetylated 909K of RIG-I in 99% purity and it clearly detects RIG-I K909Q could resolve the concerns about structure and its function.*

*We hope that Figure 6D (which is performed in RIG-I$^{-/-}$ MEF) was misunderstood as in HDAC6 knockout. We intended to show the effect of RIG-I and its mutant in the RIG-I$^{-/-}$ MEF by transient transfection and demonstrated that the RIG-I K909R has antiviral activity as a RIG-I WT while acetylation-mimic mutant is not. Furthermore, we assessed the effect of HDAC6 on RIG-I in Figure 6E, and RIG-I WT did not bind to ligand under HDAC6 deficiency unlike K909R mutant. To improve our manuscript in accordance with referee's concern, we have performed transient transfection of RIG-I WT and its mutant to HDAC6$^{+/+}$ and HDAC6$^{-/-}$ MEF. Consistent with Figure 6E, HDAC6$^{-/-}$ MEF cells transfected with RIG-I WT show the minimum of antiviral effect as control or RIG-I K909Q, but HDAC6$^{-/-}$ MEF cells transfected with RIG-I K909R show the striking antiviral*

*effect (Figure EV2). Our results suggest again that deacetylation of RIG-I through HDAC6 is essential process in antiviral signaling although additional target and pathway of HDAC6 obviously exist based on our result and other published papers.*

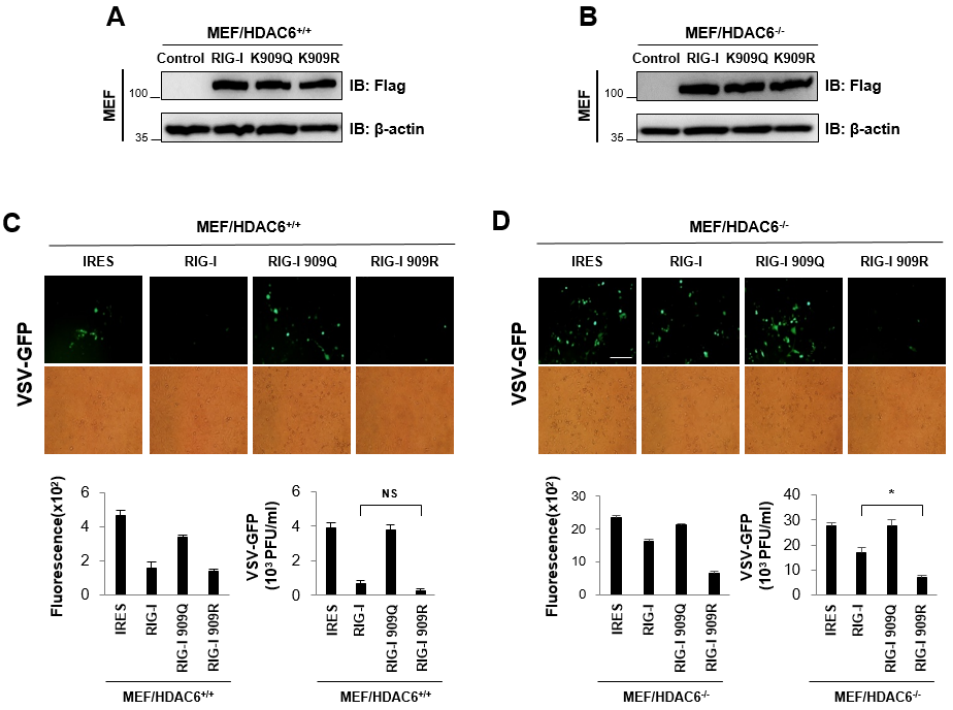



**Figure EV2**

**Comments)** 5. What is the basis for the transient nature of the HDAC6 interaction with RIG-I?

***Response)*** *We thank the reviewer for insightful question. Transient nature of innate immune response is well documented in several reviews (Ma et al, 2013; Yoneyama et al, 2008). Normally, innate immune system immediately reacts against viral invasion and regress back to normal on virus clearance status. Since overload innate immune responses can cause harmful effect to host itself, molecules in the pathway undergo various modification to be activated positively or negatively. Therefore, it is not surprising that regulation machinery of innate immune response could be transient. Likely, RIG-I is also tightly controlled by several modifications like phosphorylation, ubiquitination, acetylation and RIG-I dependent signaling is also reported as transient signal (Papon et al, 2009). Interestingly, it was also predicted that oligomerization of RIG-I could be transient (Louber et al, 2014). In addition, HDAC6 could mediate transient deacetylation of its substrate (Serrador et al, 2004).*

*As shown in our figures, ectopic expressed (Figure 6F and Figure EV3) and endogenous (Figure 6G and H) conserves acetylation of 909$^{th}$ lysine, which prevents activation of RIG-I. It suggests that RIG-I is maintained to be suppressed its sensing function by acetylation in uninfected condition. Therefore, we hypothesis that this suppression would be dissolved by HDAC6 which is a deacetylating enzyme, to trigger innate immunity in response to virus infection, and it would not be necessary to maintain the interaction after deacetylation for the homeostasis of host cells to prevent overload of RIG-I mediated signaling or conformation of RIG-I.*

*We include this discussion in page 18 Line 19-24.*

**Supporting references:**


1. Ma Z, Moore R, Xu X, Barber GN (2013) DDX24 Negatively Regulates Cytosolic RNA-Mediated Innate Immune Signaling. PLoS Pathog 9: e1003721
2. Yoneyama M, Onomoto K, Fujita T (2008) Cytoplasmic recognition of RNA. Advanced Drug Delivery Reviews 60: 841-846 **(841)**
3. Papon L, Oteiza A, Imaizumi T, Kato H, Brocchi E, Lawson TG, Akira S, Mechti N (2009) The viral RNA recognition sensor RIG-I is degraded during encephalomyocarditis virus (EMCV) infection. Virology 393: 311-318 **(315)**
4. Louber J, Kowalinski E, Bloyet L-M, Brunel J, Cusack S, Gerlier D (2014) RIG-I Self-Oligomerization Is Either Dispensable or Very Transient for Signal Transduction. PLoS ONE 9: e108770
5. Serrador JM, Cabrero JR, Sancho D, Mittelbrunn M, Urzainqui A, Sanchez-Madrid F (2004) HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 20: 417-428 **(419)**


**Comments)** 6. Why does RIG-I expression induce IFN transcription (Fig. 6F)? This result is different from many previous reports that shown that RIG-I activity is completely dependent on dsRNA.

***Response)*** *We thank the reviewer for insightful comment. We hope that Figure 6F was misunderstood as Figure 4F. RIG-I activity is completely dependent on dsRNA, but RIG-I induces IFN-transcription itself when it comes to overexpression (Cui et al, 2012; Long et al, 2014; Xia et al, 2015). Besides, as shown in many reports (Kim & Seed, 2010; Pauli et al, 2014), overexpression of CARD domain of RIG-I also enhances IFN-β luciferase activity without dsRNA stimulation. In case of Figure 6C, we have shown the slightly enhanced IFN-β luciferase activity by RIG-I expression. However, in this case, to precisely assess the function of RIG-I and its mutant about dsRNA-induced IFN transcription, we repeated the experiment several times and minimized the stimulation of its own activity.*

**Supporting references:**


1. Cui J, Li Y, Zhu L, Liu D, Songyang Z, Wang HY, Wang RF (2012) NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4. Nature immunology 13: 387-395 **(390)**
2. Long L, Deng Y, Yao F, Guan D, Feng Y, Jiang H, Li X, Hu P, Lu X, Wang H, Li J, Gao X, Xie D (2014) Recruitment of phosphatase PP2A by RACK1 adaptor protein deactivates transcription factor IRF3 and limits type I interferon signaling. Immunity 40: 515-529 **(518)**
3. Xia P, Wang S, Xiong Z, Ye B, Huang LY, Han ZG, Fan Z (2015) IRTKS negatively regulates antiviral immunity through PCBP2 sumoylation-mediated MAVS degradation.

Nature communications 6: 8132 **(4)**

4. Kim H, Seed B (2010) The transcription factor MafB antagonizes antiviral responses by blocking recruitment of coactivators to the transcription factor IRF3. Nature immunology 11: 743-750 **(744)**
5. Pauli EK, Chan YK, Davis ME, Gableske S, Wang MK, Feister KF, Gack MU (2014) The ubiquitin-specific protease USP15 promotes RIG-I-mediated antiviral signaling by deubiquitylating TRIM25. Science signaling 7: ra3 **(6)**

2nd Editorial Decision 08 December 2015

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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REFEREE REPORT

Referee #1:

I thought that the original manuscript was solid and interesting. The revised version, including multiple new experiments have improved the manuscript.

My major concerns have been addressed.