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LRRC25 inhibits type I IFN signaling by targeting ISG15-associated RIG-I for autophagic degradation

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(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 6 April 2017

Thank you again for submitting your manuscript for consideration by the EMBO Journal. I copy once more the referee reports for your information below.

Thank you also for sending a point-by-point response draft upfront, which I requested since the referees noted lack of sufficient depth and, importantly, demonstration of physiological relevance for the findings reported in your manuscript. I have now read your response and appreciate it. It seems that you are able and willing to add substantial new experimental data to add more insight into the mechanism underlying RIG-I degradation and into the potential physiological significance of your findings. Though the outcome of the experiments is unclear at this stage, I am thus happy to consider a revised version of your work. Please note that I would however need strong support from all referees on such a revised version in order to move forward here.

I should also remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The study reports on one of the mechanism's of downregulation of RIG-I signaling that involves autophagy pathway and a novel p62-dependent but ubiquitin-independent and instead of that, ISG15-dependent mechanism. The study is of interest, but is somewhat naïve and at times agnostic

of the known pathways involved in autophagy and the engagement of TBK1 directly in the autophagy progression. The significance of the particular pathway studied by the authors relative to others (e.g. autophagy has been shown previously to interfere with RIG-I signaling) needs to be assessed/established in some relevant model.

1. The study is interesting when it comes to the aspect where the authors found that degradation of RIG-I was independent of ubiquitination. This however needs to be seriously shored up experimentally.

2. The experiments with TBK-1 expression rescuing autophagic(?) degradation of RIG-I assume that it is all expression downstream of IRF-controlled genes. But, TBK-1 is also known to phosphorylate p62 and other like receptors. Have the authors considered the possibility that TBK1 in their experiments acted directly as a kinase on p62?

3. The biological significance of LRRC25-sponsored degradation of RIG-I in response to viral infection is unclear, especially relative to numerous other mechanisms previously described, and needs to be established in a relevant model, preferably in vivo.

4. ISG15 entering the scene in this work. The authors leap form TBK-1 expression to ISG15 without any consideration of other genes expressed under TBK-1 effects. They just looked at ISG15, because of one prior publication. Is this enough to focus on ISG15?

5. In Fig. 1A, based on the screening results, besides LRRC25, LRRC42 and LRRC46 also inhibit RLR-mediated type I IFN signaling pathway, do LRRC42 and LRRC46 interact with ISG15? Or interact with LRRC25 as a complex?

6. In Fig. 1B, LRRC25 protein level also need to be analyzed under intracellular poly low molecular weight and IFN-β (the same conditions as Fig. 1C), and IFN-1 level should be detected under treatment during 24h.

7. In Fig.1D, Overexpression of RIG-I(N) or blocking proteasome -dependent degradation stabilized LRRC25; does RIG-I(N) affect proteasome degradation function or does IFN activation block the proteasome -dependent degradation? They need an experiment to exclude that.

8. Does LRRC25KO affect transcription levels of RIG-I?

9. In Fig 3A, does RIG-I have the same effect as RIG-I (N)?

10. GST-Pulldown essay is necessary to test whether the interactions of RIG-I with ISG15 or ISG15 with LRRC25 are direct or not.

11. According to Fig 4A, RIG-I(N) l were reduced with increasing LRRC25 protein level, but in Fig 3D WCL, the increasing LRRC25 protein level is associated with increased RIG-I level.

12. In Fig4 E, the Flag control is necessary for IP.

13. To prove that LRRC25 degrades RIG-1 through the autophagosome pathway, knockdown LC3 or bafilomycin A1 treatment is required.

14. In Figure 5E, in p62ko cell line, Flag-RIG-I(N) was still degraded by myc-LRRC25, since LRRC25 is degraded by proteasome-dependent degradation (Fig 1D). So is it possible that LRRC25 degrades RIG-I not only through the autophagy pathway? Need more experiments to exclude this.

15. Does ISG15 KO affect the interaction of LRRC25 and p62?

16. Confocal images of are needed to prove the colocalization/interaction of LRRC25 and P62, ISG15 with RIG-I and LRRC25, LRRC25 and RIG-I.

17. At what time does ISG15 come into play in natural sequence of events during viral infection?

Minor:

1. Abstract: "is a key" not "as a key"
2. 2. "LRRC25 degrades RIG-I through autophagosome pathway" - "autophagosome" is not a pathway.

Referee #2:

In this work Du et al have investigated the role of LRRC proteins in regulation of the RIG-I pathway. They identify LRRC25 to be a negative regulator of the pathway, and to act by targeting ISG15-associated RIG-I for autophagic degradation in a p62-dependent manner. This is an interesting story, and the data presented are generally strong, and do support the conclusions drawn. However, key data are still missing to fully consolidate the story.

MAJOR POINTS
1. Most results are based on biochemical approached, with 293T-cell-based systems being the preferred model system. The authors should complement their data with confocal microscopy results. As a minimum, the subcellular localizations of LRRC25, RIG-I, and p62 over time after stimulation should be evaluated.

2. Is the effect of LRRC25 on innate immune responses specific for the RIG-I pathway? It should be tested how the cGAS-STING, and TLR3-TRIF pathways are influenced by LRRC25. Even more importantly, the impact of LRRC25 on MDA5-mediated IFN expression should be tested.

3. Figure 4. The inhibitor data should be complemented with data from genetic models. Beclin1 KO cells is not sufficient. Atg5 KO cells should also be tested

4. I do not find any data with primary human cells in the manuscript. This is important in order to be able to evaluate the physiological importance of the finding.

5. The work would gain if the authors can demonstrate LRRC25 being involved in regulation of the IFN response to a human pathogenic virus (e.g. influenza A virus).

6. The weakest part is the proposed mechanism of RIG-I degradation. More details are required on how ISG15 and LRRC25 assemble a complex that bridge RIG-I to p62. The genetic data provided are compelling, but the mechanistic part is underdeveloped.

Referee #3:

Du et al. reported that LRRC25 served as a RIG-I negative regulator upon activation. The mechanism presented here is that LRRC25 promoted RIG-I's interaction with p62, which directed RIG-I to autophagy and subsequent degradation. The negative regulation was strictly dependent on ISG15 conjugation. Most of the data are clear-cut and support the author's conclusion. The findings that ISGylation of RIG-I is directed by LRRC25 for autophagy-mediated degradation is novel. However, the ISGylation of RIG-I was previously shown to be a prerequisite for RIG-I degradation. There are several questions that need to be addressed.

While the data on knockdown/KO of LRRC25 are clear and solid (figure 2), the overexpression of LRRC25 on RIG-I is somewhat confounding. MG132 was used in almost all overexpression systems. The author needs to explain the reason of including this broad-spectrum proteasome inhibitor (Figure 1G, Figure 4B, Figure 4G, Figure 4H, Figure 6B, etc). Artificial overexpression system, together with the treatment of MG132, likely creates a condition that is not physiologically relevant. Additionally, the author failed to include any controls without MG132 treatment. If the phenotype (e.g., inhibition of IRF signaling, RIG-I degradation) could only be observed by increasing the protein amount of the already overexpressed LRRC25 via MG132 treatment (or manipulating RIG-I ISGylation by MG132), the biological significance of LRRC25 in regulating

RIG-I ISGylation, degradation and signaling remains questionable.
Figure 4C did not provide credence to the conclusion that LRRC25 promotes the degradation of RIG-I. As Figure 2 already showed elevated IFNb upon LRRC25 knockdown, the up-regulated protein level of RIG-I rather could be explained by the significantly elevated IFN production. This is consistent with that RIG-I is interferon-inducible.

The hypothesis of autophagosome degradation of RIG-I was only supported by biochemistry data on p62 interaction and chemical treatment. To further endorse their hypothesis, authors will need immunostainings to show the formation of autophagosomes upon viral infection and the localization of Rig-I in the autophagosomes under normal and LRRC25-depleted conditions.

Figure 6B,C,D,E,G all based on overexpression of both RIG-I-N and LRRC25. The effect of LRRC25 on endogenous RIG-I is highly recommended.

1st Revision - authors' response 21 July 2017

I would like to thank you and the reviewers for the positive and thoughtful comments and suggestions regarding our manuscript. We have performed additional experiments and provided new data (17 new figures incorporated in the main figure and text, supplemental figures and 1 figure in the response letter) in accordance with the reviewers' suggestions. For the convenience, we have numbered all of the suggestions in response to each question in this letter. A point-to point response to the reviewers' concerns is included below.

Response to the comments of Reviewer #1
Comment 1*. The study is interesting when it comes to the aspect where the authors found that degradation of RIG-I was independent of ubiquitination. This however needs to be seriously shored up experimentally.*
Response: In the original manuscript, we showed that p62 DUBA (lacking ubiquitin-binding domain) still interacts with RIG-I for autophagic degradation (Fig 5B), suggesting that the interaction between RIG-I and p62 is independent on poly-ubiquitination. In addition, we showed that RIG-I-N (K164/172R), the RIG-I mutant lacking two main K63-linked ubiquitination sites, could still be mediated for the degradation by LRRC25 (original Appendix Fig S5A). These experiments indicate that the degradation of RIG-I mediated by LRRC25 is independent of ubiquitination. To further confirm our conclusion, we mutated all the known ubiquitination sites on RIG-I (N) (Lys 48, 99, 154, 164, 169, 172, 181, 190) to generate RIG-I (N) (8KR) mutant, and found that LRRC25 could still degrade RIG-I (N) (8KR) in the presence of IFN-b (**New Fig 1, related to Fig EV4G in the manuscript**), indicating that the degradation of RIG-I promoted by LRRC25 was independent of RIG-I ubiquitination.

(Figure for referees not shown)

Comment 2*. The experiments with TBK-1 expression rescuing autophagic(?) degradation of RIG-I assume that it is all expression downstream of IRF-controlled genes. But, TBK-1 is also known to phosphorylate p62 and other like receptors. Have the authors considered the possibility that TBK1 in their experiments acted directly as a kinase on p62?*
Response: According to the reviewer's suggestions, we treated the cells with IFN-b to activate downstream ISG genes to exclude the possibility that TBK1 acts directly as a kinase on p62. We found that LRRC25 could still promote the degradation of RIG-I (N) in the presence of IFN-b in MAVS KO cells (**New Fig 2, related to Fig 6E in the manuscript**), which further suggests that the activation of type I IFN signaling is an essential signal for autophagic degradation of RIG-I.

(Figure for referees not shown)

Comment 3. *The biological significance of LRRC25-sponsored degradation of RIG-I in response to viral infection is unclear, especially relative to numerous other mechanisms previously described, and needs to be established in a relevant model, preferably in vivo.*
Response: As we discussed in our original manuscript, several studies have reported that RIG-I could be degraded through proteasomal pathway by several E3 ligases such as RNF125, CHIP and Siglec-G/c-Cbl (Arimoto et al, 2007; Chen et al, 2013; Zhao et al, 2016), here we firstly identified that the stability of RIG-I could also be controlled through autophagic pathway. To show the biological significance of LRRC25-sponsored degradation of RIG-I in response to viral infection, we isolated human peripheral blood mononuclear cells (PBMCs) and knocked down endogenous LRRC25 to evaluate the physiological importance of LRRC25 upon influenza A (H1N1) infection. As expected, we found that knockdown of endogenous *LRRC25* increased the phosphorylation of endogenous IRF3 after stimulated by H1N1 in PBMCs (**New Fig 3A, related to Fig 2H in the manuscript**). Furthermore, qPCR analysis showed that the deficiency of LRRC25 enhanced the transcription of *IFN-b*, *IFIT1* and *IFIT2* (**New Fig 3B and 3C, related to Fig 2I and 2J in the manuscript**). Taken together, these data indicate that LRRC25 plays a significant role in antiviral immunity in human primary cells.

(Figure for referees not shown)

Comment 4. *ISG15 entering the scene in this work. The authors leap from TBK-1 expression to ISG15 without any consideration of other genes expressed under TBK-1 effects. They just looked at ISG15, because of one prior publication. Is this enough to focus on ISG15?*
Response: Firstly, as compared to wild type 293T cells, we observed no degradation of RIG-I (N) and FL RIG-I in *MAVS* KO 293T cells, indicating that simply exposure of RIG-I CARD domain is not enough to initiate LRRC25-mediated RIG-I degradation (Fig 6B and 6C), and the activation of type I IFN signaling is an essential signal for degradation of RIG-I. Secondly, it has been reported that RIG-I can be modified by ubiquitin and ISG15 (Davis & Gack, 2015; Kim et al, 2008). Since we showed that p62 DUBA (lacking ubiquitin-binding domain) still interacted with RIG-I for autophagic degradation (Fig 5B), suggesting that the interaction between RIG-I and p62 is independent on poly-ubiquitin signal. In addition, we showed that RIG-I-N (8KR) mutant, lacking all the known ubiquitination sites, could still be mediated for degradation by LRRC25 (**Fig EV4G**). All these data excluded the possibility that ubiquitination of RIG-I is involved in the degradation of RIG-I mediated by LRRC25. More importantly, we observed that LRRC25 could promote the degradation of RIG-I (N) in the presence of IFN-b in MAVS KO cells (**New Fig 2, related to Fig 6E in the manuscript**), suggesting that downstream ISGs might contribute to the degradation of RIG-I, thus we proposed that ISG15 may participate in the degradation of RIG-I promoted by LRRC25. Surprisingly, we observed that the degradation of RIG-I (N) mediated by LRRC25 was abrogated in the absence of ISG15 (Fig 6H). Taken together, we concluded that ISG15 is the major contributor in the degradation of RIG-I (N) mediated by LRRC25.

Comment 5. *In Fig. 1A, based on the screening results, besides LRRC25, LRRC42 and LRRC46 also inhibit RLR-mediated type I IFN signaling pathway, do LRRC42 and LRRC46 interact with ISG15? Or interact with LRRC25 as a complex?*
Response: To address this question, we performed the immunoprecipitation experiments to test the interaction between LRRC42, LRRC46 and ISG15 as well as LRRC25. We found that LRRC42 but not LRRC46 could interact with LRRC25 and ISG15 (**New Fig 4A and 4B**). However, we found that LRRC42 had no effects on the protein level of RIG-I (N) (**New Fig 4C**). LRRC42 might exert other functions with LRRC25, but not involved in the RIG-I degradation. Taken together, these results suggest that LRRC42 simply interacts with LRRC25 and ISG15, but has no effects on the protein level of RIG-I.

(Figure for referees not shown)

Comment 6. *In Fig. 1B, LRRC25 protein level also need to be analyzed under intracellular poly (I:C) low molecular weight and IFN-β (the same conditions as Fig. 1C), and IFN-b level should be detected under treatment during 24h.*

Response: To address this question, we performed immunoblot analysis to evaluate the expression of LRRC25 under intracellular poly(I:C) low molecular weight and IFN-β, and found that the LRRC25 protein level was up-regulated under intracellular poly(I:C) low molecular weight and IFN-β treatment (**New Fig 5A, related to Fig 1B, 1C and 1D in the manuscript**). However, the mRNA level of LRRC25 were not increased after treatments of intracellular (IC) poly(I:C) low molecular weight (LMW) (a synthetic ligand for RIG-I), IFN-b or VSV-eGFP infection (**New Fig 5B, related to Fig EV1A in the manuscript**). Together, these results indicate that LRRC25 is up-regulated by type I IFNs at protein level.

(Figure for referees not shown)

Comment 7*. In Fig.1D, Overexpression of RIG-I (N) or blocking proteasome -dependent degradation stabilized LRRC25; does RIG-I (N) affect proteasome degradation function or IFN activation blocks the proteasome -dependent degradation? Need experiment to exclude that.*
Response: To address this question, we tested whether RIG-I (N) can block the proteasome degradation of TBK1 promoted by USP38 (Lin et al, 2016), and found that ectopic expression of RIG-I (N) could not block the proteasome degradation of TBK1 mediated by USP38 (**New Fig 6, related to Fig EV1C in the manuscript**), which indicates that the stabilization of LRRC25 mediated by overexpression of RIG-I (N) is specific, and further excludes the possibility that RIG-I (N) blocks the proteasome-dependent degradation.

(Figure for referees not shown)

Comment 8*. Does LRRC25KO affect transcription levels of RIG-I?*
Response: RIG-I is an ISG gene, which is up-regulated by type I IFNs. As shown in Figure 2, LRRC25 KO can elevate the transcription of IFN-b and downstream ISGs. Thus, LRRC25 KO can up-regulate the transcription levels of RIG-I upon viral infection.

Comment 9*. In Fig 3A, does RIG-I have the same effect as RIG-I (N)?*
Response: As the reviewers suggested, we overexpressed RIG-I and treated the cells with IC poly(I:C) and analyzed whether LRRC25 could affect the activation of ISRE induced by RIG-I. As we presented in **New Fig 7** (**related to Fig EV3B in the manuscript**), LRRC25 markedly inhibited RIG-I-mediated ISRE-luc activation after IC poly (I:C) stimulation. However, in the absence of stimulation, we only detected a slight activation of ISRE-luc, which was not affected by LRRC25.

(Figure for referees not shown)

Comment 10. *GST-Pulldown assay is necessary to test whether the interactions of RIG-I with ISG15 or ISG15 with LRRC25 are direct or not.*
Response: We have constructed HA-, Flag- and GST-tagged-ISG15 and tested the interaction between RIG-I, LRRC25 and ISG15 previously. However, we found that the tag on ISG-15 may affect its binding to RIG-I, that's why we used untagged-ISG15 in the entire study. Therefore, the GST-Pulldown assay cannot be done in this study. In addition, in our work, we identified ISG15 as a novel mediator, which bridges RIG-I to LRRC25 for degradation. Whether their interactions are direct or not, is not a critical issue in this work.

Comment 11*. According to Fig 4A, RIG-I (N) were reduced with increasing LRRC25 protein level, but in Fig 3D WCL, the increasing LRRC25 protein level is associated with increased RIG-I level.*
Response: We respectfully disagree on this point. It is well-known that RIG-I is an ISG gene and the mRNA level of RIG-I will be up-regulated upon viral infection. We have demonstrated that the expression of LRRC25 will be up-regulated upon viral infection (Fig 1B). So, it is reasonable that the increasing endogenous LRRC25 protein level is associated with increased endogenous RIG-I level upon viral infection in THP-1 cells (Fig 3D). More importantly, we have shown that upon IC ploy(I:C) treatment, the degradation of RIG-I is blocked in LRRC25 KO cells in the presence of

CHX, which blocked the synthesis of RIG-I protein induced by type I IFNs (**Fig EV5C**), indicating that LRRC25 negatively regulates RIG-I stability.

Comment 12. *In Fig4 E, the Flag control is necessary for IP.*
Response: Here the reviewer may misunderstand our work. We did not perform IP in Fig. 4 E. If the reviewer means Fig 3E, the first lane of Fig 3E is the negative control (the lysates from the cells transfected with empty vector instead of RIG-I or its mutants).

Comment 13. *To prove that LRRC25 degrades RIG-1 through autophagosome pathway, knockdown LC3 or bafilomycin A1 treatment is required.*
Response: As the reviewer suggested, we examined whether bafilomycin A1 could restore the degradation of RIG-I (N) promoted by LRRC25. As presented in **New Fig 8** (**related to Fig EV4E in the manuscript**), ectopic expression of LRRC25 failed to mediate the degradation of RIG-I (N) in the presence of autophagy inhibitor bafilomycin A1, indicating that LRRC25 promotes the degradation of RIG-I through autophagosome.

(Figure for referees not shown)

Comment 14. *In Figure 5E, in p62ko cell line, Flag-RIG-I (N) was still degraded by myc-LRRC25, since LRRC25 is degraded by proteasome-dependent degradation (Fig 1D), So is it possible that LRRC25 degrades RIG-I not only through autophagy pathway? Need more experiments to exclude this.*
Response: We respectfully disagree with this comment that in p62 KO cell line, Flag-RIG-I (N) was still degraded by myc-LRRC25. We have repeated this experiment for at least three times and analyzed the bands density in WT and p62 KO cell line (**New Fig 9, related to Fig 5E and Fig EV4H in the manuscript**), which suggests the deletion of p62 can totally rescue the degradation of RIG-I (N) mediated by LRRC25, indicating that LRRC25 promotes the degradation of RIG-I (N) only through autophagy.

(Figure for referees not shown)

Comment 15. *Does ISG15 KO affect the interaction of LRRC25 and p62?*
Response: To address this question, we performed the immunoprecipitation experiment and found that LRRC25 could still interact with p62 in ISG15 KO cell line (**New Fig 10, related to Fig EV5H in the manuscript**).

(Figure for referees not shown)

Comment 16. *Confocal images of are needed to prove the colocalization/interaction of LRRC25 and P62, ISG15 with RIG-I and LRRC25, LRRC25 and RIG-I.*
Response: As the reviewer suggested, we performed the confocal analysis to evaluate the colocalization of RIG-I, ISG15, LRRC25 and p62 in HeLa cells. As expected, confocal microscopy showed that RIG-I, ISG15, LRRC25 and p62 formed puncta upon intracellular (IC) poly(I:C) stimulation (**New Fig 11, related to Fig 7H in the manuscript**).

(Figure for referees not shown)

Comment 17. *At what time does ISG15 come into play in natural sequence of events during viral infection?*
Response: Upon RNA virus infection, RIG-I triggers the activation of type I IFN signaling and up-regulates ISG15. ISG15 then plays a role in the antiviral response. In our original manuscript, we have shown that after viral infection, ISG15 bridges the interaction between RIG-I and LRRC25,

and the deficiency of ISG15 blocks the association between RIG-I and LRRC25. To further confirm the acting sequence of ISG15 after viral infection, we performed immunoprecipitation experiments to evaluate the endogenous interaction between RIG-I, ISG15, LRRC25 and p62 at certain time points during the viral infection. Consistent with our previous data, the immunoprecipitation results showed that after viral infection, RIG-I firstly interacted with ISG15, and then the ISG15-associated RIG-I interacted with LRRC25 and p62 (**New Fig 12, related to Fig 7G in the manuscript**).

(Figure for referees not shown)

Minor:
Comment 18. *Abstract: "is a key" not "as a key"*
Response: We sincerely regret for the inadvertent mistake in writing this sentence. We have corrected this sentence in the revised manuscript.

Comment 19. *"LRRC25 degrades RIG-I through autophagosome pathway" - "autophagosome" is not a pathway.*
Response: We sincerely regret for the inadvertent mistake in writing this sentence. We have corrected this sentence in the revised manuscript.

Response to the comments of Reviewer #2
Comment 1. *Most results are based on biochemical approached, with 293T-cell-based systems being the preferred model system. The authors should complement their data with confocal microscopy results. As a minimum, the subcellular localizations of LRRC25, RIG-I, and p62 over time after stimulation should be evaluated.*
Response: Regarding the subcellular localizations of LRRC25, RIG-I and p62 over time after stimulation, please see above the response to the comment #16 of reviewer #1.

Comment 2. *Is the effect of LRRC25 on innate immune responses specific for the RIG-I pathway? It should be tested how the cGAS-STING, and TLR3-TRIF pathways are influenced by LRRC25. Even more importantly, the impact of LRRC25 on MDA5-mediated IFN expression should be tested.*
Response: Firstly, we have already showed that LRRC25 had no effect on the type I IFN signaling pathway induced by c-GAS (Fig EV1E). Secondly, we have found that LRRC25 can promote the degradation of MDA5 and down-regulate the activation of type-I IFN signal induced by MDA5 (**New Fig 13A and 13B, related to Fig EV3C and EV4C in the manuscript**). For TLR3-TRIF pathway, we performed the luciferase assay and found that LRRC25 had no effect on the activation of ISRE induced by TLR3 after poly (I:C) stimulation (**New Fig 13C , related to Fig EV1F in the manuscript**).

(Figure for referees not shown)

Comment 3. *Figure 4. The inhibitor data should be complemented with data from genetic models. Beclin1 KO cells is not sufficient. Atg5 KO cells should also be tested.*
Response: As the reviewer suggested, we co-expressed RIG-I (N) and LRRC25 in WT and Atg5 KO cells. As expected, we found that the degradation of RIG-I (N) mediated by LRRC25 could be blocked in Atg5 KO cells (**New Fig 14, related to Fig 4J in the manuscript**).

(Figure for referees not shown)

Comment 4. *I do not find any data with primary human cells in the manuscript. This is important in order to be able to evaluate the physiological importance of the finding.*
Response: We used primary human cells – PBMCs to perform the experiments as the reviewer suggested, please see above the response to the comment 3 of reviewer #1.

Comment 5. *The work would gain if the authors can demonstrate LRRC25 being involved in regulation of the IFN response to a human pathogenic virus (e.g. influenza A virus).*
Response: We used the influenza A virus to perform the experiments as the reviewer suggested, please see above the response to the comment #3 of reviewer #1.

Comment 6. *The weakest part is the proposed mechanism of RIG-I degradation. More details are required on how ISG15 and LRRC25 assemble a complex that bridge RIG-I to p62. The genetic data provided are compelling, but the mechanistic part is underdeveloped.*
Response: As we showed in Fig 7, ISG15 bridged the association between RIG-I and LRRC25 through interacting with RIG-I and LRRC25. Furthermore, in Fig 5C, we showed that LRRC25 can interact with p62. To further investigate the assembling sequence, we performed immunoprecipitation experiments to evaluate the endogenous interaction between RIG-I, ISG15, LRRC25 and p62 at certain time points during the viral infection. Consistent with our previous data, the immunoprecipitation results showed that after viral infection, RIG-I firstly interacted with ISG15, and then the ISG15-associated RIG-I interacted with LRRC25 and p62 (**New Fig 12, related to Fig 7G in the manuscript**).

Response to the comments of Reviewer #3
Comment 1. *While the data on knockdown/KO of LRRC25 are clear and solid (figure 2), the overepression of LRRC25 on RIG-I is somewhat confounding. MG132 was used in almost all the overexpression system. The author needs to explain the reason of including this broad-spectrum proteasome inhibitor (Figure 1G, Figure 4B, Figure 4G, Figure 4H, Figure 6B, etc). Artificial overexression system, together with the treatment of MG132, likely creates a condition that is not physiologically relevant. Additionally, the author failed to include any controls without MG132 treatment. If the phenotype (e.g., inhibition of IRF signaling, RIG-I degradation) could only be observed by increasing the protein amount of the already overexpressed LRRC25 via MG132 treatment (or manipulating RIG-I ISGylation by MG132), the biological significance of LRRC25 in regulating RIG-I ISGylation, degradation and signaling remains questionable.*
Response: Actually, we firstly observed the degradation of RIG-I mediated by LRRC25 after treatment with intracellular poly (I:C) LMW (**New Fig 15A, related to Fig EV4A in the manuscript and New Fig 15B**) or overexpressed TBK1 without adding MG132 (**New Fig 15C**). However, we also noticed that LRRC25 could also be stabilized when type I IFN signaling is activated. As showed in Fig1E and Fig EV1B, type I IFN signaling stabilized LRRC25 by blocking its proteasome-dependent degradation, and the proteasome inhibitor MG132, but not the lysosome inhibitor NH_4Cl could stabilize LRRC25 and diminish the difference of LRRC25 protein level with or without type I IFN activation. To exclude the possibility that the less amount of RIG-I is due to more LRRC25 upon stimulation, we used MG132 to make the protein levels of LRRC25 equal among different groups to compare its ability for RIG-I degradation during type I IFN activation. Taken together, our data suggest that MG132 does not affect the function of LRRC25 in mediating RIG-I degradation.

(Figure for referees not shown)

Comment 2. *Figure 4C did not provide credence to the conclusion that LRRC25 promotes the degradation of RIG-I. As Figure 2 already showed elevated IFN-b upon LRRC25 knockdown, the up-regulated protein level of RIG-I rather could be explained by the significantly elevated IFN production. This is consistent with that RIG-I is interferon-inducible.*
Response: Since RIG-I is an ISG gene, it is indeed difficult to make the conclusion that LRRC25 promotes the degradation of RIG-I in this system. However, in our original manuscript, we showed that LRRC25 could promote the degradation of RIG-I (Fig 4 and 6). Moreover, we generated another system to analyze the degradation of endogenous RIG-I mediated by LRRC25. We ectopically expressed ISG15 in 293T cells and pre-treated the cells with cycloheximide (CHX), which blocks the protein synthesis, including RIG-I, followed by IC poly(I:C) treatment. We found that endogenous RIG-I was sharply reduced after treatment of IC poly(I:C), but no such dramatic reduction in RIG-I was observed in LRRC25 knockout cells (**New Fig 16, related to Fig EV5C in the manuscript**), which further confirms that LRRC25 mediates the degradation of endogenous RIG-I.

(Figure for referees not shown)

Comment 3*. The hypothesis of autophagosome degradation of RIG-I was only supported by biochemistry data on p62 interaction and chemical treatment. To further endorse their hypothesis, authors will need immunostaining to show the formation of autophagosome upon viral infection and the localization of RIG-I in the autophagosome under normal and LRRC25-depleted conditions.*
Response: As the reviewer suggested, we performed the confocal analysis to show the co-localization between RIG-I and LC3 upon IC poly(I:C) treatment in LRRC25 deficient cells. We found LRRC25 deficiency reduced the co-localization between RIG-I and LC3 (**New Fig 17，related to Fig 4K in the manuscript**).

(Figure for referees not shown)

Comment 4*. Figure 6B, C, D, E, G all based on overexpression of both RIG-I-N and LRRC25. The effect of LRRC25 on endogenous RIG-I is highly recommended.*
Response: As the reviewer suggested, we repeated the experiments in Fig 6C and original 6G (**related to Fig 6H in the manuscript**) to examine the effect of LRRC25 on endogenous RIG-I upon IC poly (I:C) LMW stimulation in MAVS KO and ISG15 KO cells. Consistent with our previous data, we observed no degradation of endogenous RIG-I mediated by LRRC25 in MASV KO cells. However, the ability of LRRC25 to degrade RIG-I was restored in the presence of IFN-b in *MAVS* KO cells (**New Fig 18A, related to Fig 6F in the manuscript**). Furthermore, the degradation of endogenous RIG-I was abolished in ISG15 KO cells (**New Fig 18B, related to Fig 6I in the manuscript**). Other experiments to address this issue can be found in comment #2 to reviewer #3.

(Figure for referees not shown)

References cited:

Arimoto K, Takahashi H, Hishiki T, Konishi H, Fujita T, Shimotohno K (2007) Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 7500-7505

Chen W, Han C, Xie B, Hu X, Yu Q, Shi L, Wang Q, Li D, Wang J, Zheng P, Liu Y, Cao X (2013) Induction of Siglec-G by RNA viruses inhibits the innate immune response by promoting RIG-I degradation. *Cell* **152:** 467-478

Davis ME, Gack MU (2015) Ubiquitination in the antiviral immune response. *Virology* **479-480:** 52-65

Kim MJ, Hwang SY, Imaizumi T, Yoo JY (2008) Negative feedback regulation of RIG-I-mediated antiviral signaling by interferon-induced ISG15 conjugation. *Journal of virology* **82:** 1474-1483

Lin M, Zhao Z, Yang Z, Meng Q, Tan P, Xie W, Qin Y, Wang RF, Cui J (2016) USP38 Inhibits Type I Interferon Signaling by Editing TBK1 Ubiquitination through NLRP4 Signalosome. *Molecular cell* **64:** 267-281

Zhao K, Zhang Q, Li X, Zhao D, Liu Y, Shen Q, Yang M, Wang C, Li N, Cao X (2016) Cytoplasmic STAT4 Promotes Antiviral Type I IFN Production by Blocking CHIP-Mediated Degradation of RIG-I. *Journal of immunology (Baltimore, Md : 1950)* **196:** 1209-1217

Thanks for the opportunity to improve the quality of this manuscript by incorporating these suggested changes.

2nd Editorial Decision 14 September 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees whose comments are shown below.

As you will see, the referees appreciate the revised work. However, some issues remain, and it is important to address these to make your manuscript a strong candidate for publication here. I would thus like to ask you to address the remaining concerns of the reviewers. Importantly, better images with higher resolution need to be provided for the confocal microscopic analyses (figure 4K and 7H), and please pay specific attention to address the points 3, 4, 7, and 8 that referee #3 lists.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In general, the authors have addressed a number of questions and criticisms, sometimes experimentally and sometimes arguing their points.

A major conceptual and technical issue that remains is the one of how ISG15 works here. This has not been fully clarified (for example, the authors state that tags prevent demonstrations or testing of direct interactions, which is highly unusual). This reviewer considers this a major point but it seems that it has been brushed aside and minimized.

The microscopy images provided in the revision are unfortunately of exceptionally poor quality and show a few blobs in some cells. If the editors decide to move forward with this study, in the interest of EMBO J standards, it would be imperative to take a careful look at these images before proceeding further.

Referee #2:

This reviewer thinks the authors have done a great job to comply with the points I raised in my review. I am now convinced - based on the PBP response and the data in that letter. The only thing that confuses me is that the authors talk about figure numbers up to 18 in the PBP response, and I do not find that in the submitted material. It is essential that the new data genereted in revision are shown in the published paper.

Referee #3:

This is a revised manuscript that characterized the role of LRRC25 in regulating RIG-I expression via autophagic degradation pathway. The authors have carefully addressed questions with experiments. The quality of the manuscript is greatly improved. There are a few more comments that may be helpful for the author.

1. Most of the manuscript entails 293T cells with overtly expressing exogenous proteins. Although we all believe that 293T cells are convenient for transient protein expression, immune response may not be the best studied in 293T cells. Rather, the usage of THP-1 and other immune-proficient cells that the author has used will certainly complement and signify the biological relevance of the

author's findings derived from 293T cells. A few more places that the author can strengthen on this.
2. The advantage of VSV is the viral titer can be determined by plaque assay, when possible, the author is recommended to apply this method to quantify the yield of VSV in experiments that GFP was measured by flow cytometry.
3. The intracellular low molecular weight poly(I:C) the author used lacks description. More detail concerning this important reagent certainly will help the reader to understand the manuscript and potentially using this reagent in related experiments.
4. In figure 3C, the endogenous LRRC25 appears to be a doublet. Are both species LRRC25 in this precipitated immunoblot? The whole cell lysate that was analyzed in Figure 6D appears to be a single band. This requires further clarification.
5. The CARD domain appears to be the LRRC25-binding domain within RIG-I. For specificity experiment, it would be interesting to determine whether LRRC25 interacts with CARD of MDA5 and MAVS.
6. The LRRC25 knockout cells had significantly higher level of RIG-I protein, without altered mRNA level? The knockout cells are very important reagent, the author could use more of these cells to validate more of the autophagic degradation of RIG-I.
7. What do we know about the tissue specific expression of LRRC25, in relation to RIG-I expression, rather than cGAS for example.
8. The discussion deserves to include a brief summary of the negative regulatory mechanisms controlling RIG-I in signaling output. These include phosphorylation, ubiquitination-mediated degradation and more recently described deamidation by viral factors.

2nd Revision - authors' response 29 October 2017

I would like to thank you and the reviewers for the positive and thoughtful comments and suggestions regarding our manuscript. We have performed additional experiments and provided new data (3 new figures incorporated in the main and supplemental figures, and 1 figure in the response letter) in accordance with the reviewers' suggestions. A point-to point response to the reviewers' concerns is included below.

Response to the comments of Reviewer #1
Comment 1. *A major conceptual and technical issue that remains is the one of how ISG15 works here. This has not been fully clarified (for example, the authors state that tags prevent demonstrations or testing of direct interactions, which is highly unusual). This reviewer considers this a major point but it seems that it has been brushed aside and minimized.*
Response: As we mentioned in our previous response letter to the reviewers' concerns, we have constructed HA-, Flag- and GST-tagged-ISG15 and tested the interaction between RIG-I, LRRC25 and ISG15. However, because the molecular weight of ISG15 is too small, we found that the tag on ISG-15 may affect its binding to RIG-I. That's why we used untagged-ISG15 in the entire study. Therefore, the GST-pull down assay suggested by reviewer could not be done. We did not want the reviewer that we brushed the good idea aside, instead, we have tried our best, but tagged ISG15 altered its binding ability for RIG-I. Furthermore, we identified ISG15 as a novel mediator to bridge RIG-I to LRRC25 for degradation, but whether their interactions are direct or indirect might not be a critical issue.

Comment 2. *The microscopy images provided in the revision are unfortunately of exceptionally poor quality and show a few blobs in some cells. If the editors decide to move forward with this study, in the interest of EMBO J standards, it would be imperative to take a careful look at these images before proceeding further.*
Response: We sincerely regret for the inadvertent mistake in exporting these images. As we had set the wrong dpi of exported images, the resolution of the confocal images was compressed by AI software. As the editor suggested above, we have provided the images with higher resolution (**New Fig 1A and 1C**). In addition, we provided more images with higher resolution to support our conclusion in PBP response letter (**New Fig 1B and 1D, related to Fig 4K and 7H in the manuscript**).

(Figure for referees not shown)

Response to the comments of Reviewer #2

This reviewer thinks the authors have done a great job to comply with the points I raised in my review. I am now convinced - based on the PBP response and the data in that letter. The only thing that confuses me is that the authors talk about figure numbers up to 18 in the PBP response, and I do not find that in the submitted material. It is essential that the new data generated in revision are shown in the published paper.

Response: We had already added the new figures to our revised edition (see Fig EV4G, Fig 6E, Fig 2H, Fig 2I, Fig 2J, Fig 1B, Fig 1C, Fig 1D, Fig EV1A, Fig EV1C, Fig EV3B, Fig EV4E, Fig 5E, Fig EV4H, Fig EV5H, Fig 7H, Fig 7G, Fig EV3C, Fig EV4C, Fig EV1F, Fig 4J, Fig EV4A, Fig EV5C, Fig 4K, Fig 6F, Fig 6I).

Response to the comments of Reviewer #3

Comment 1. *Most of the manuscript entails 293T cells with overtly expressing exogenous proteins. Although we all believe that 293T cells are convenient for transient protein expression, immune response may not be the best studied in 293T cells. Rather, the usage of THP-1 and other immune-proficient cells that the author has used will certainly complement and signify the biological relevance of the author's findings derived from 293T cells. A few more places that the author can strengthen on this.*

Response: Firstly, in Fig 2, we had shown the biological significance of LRRC25-sponsored degradation of RIG-I in response to viral infection in THP-1 cells as well as PBMCs, which are primary immune-proficient cells. Furthermore, in Fig 3C, 3D, 7G and 7F, we had shown that LRRC25, ISG15 and p62 could interact with endogenous RIG-I and promoted the degradation of RIG-I in THP-1 cells. Taken together, these data indicate that LRRC25 plays a significant role in antiviral immunity in immune-proficient cells.

Comment 2. *The advantage of VSV is the viral titer can be determined by plaque assay, when possible, the author is recommended to apply this method to quantify the yield of VSV in experiments that GFP was measured by flow cytometry.*

Response: Firstly, in Fig 1I, 2F and EV2C, we analyzed the effect of LRRC25 on the antiviral immunity. The fluorescence microscopy analyses and the flow cytometry analyses indicated that LRRC25 markedly inhibited the type I IFN response and antiviral immunity. Furthermore, we and others have used the flow cytometry to analyze viral infection and immunity related to VSV-eGFP (Cui et al, 2012; Kondratowicz et al, 2013; Xiao et al, 2015). Taken together, the results in fluorescence microscopy and flow cytometry analyses highly indicated that LRRC25 markedly inhibited the type I IFN response and antiviral immunity.

Comment 3. *The intracellular low molecular weight poly(I:C) the author used lacks description. More detail concerning this important reagent certainly will help the reader to understand the manuscript and potentially using this reagent in related experiments.*

Response: As the reviewer suggested, we have added the description of intracellular low molecular weight poly(I:C) in the "Materials and Methods" section as followed: "It has been reported that poly(I:C)- low molecular weight (LMW) is a specific ligand for RIG-I but not MDA5 (Kato et al, 2008; Takeuchi et al, 2010). In this study, poly(I:C)- LMW was purchased as a RIG-I ligand from invivogen (Catalog # tlrl-picwlv)."

Comment 4. *In figure 3C, the endogenous LRRC25 appears to be a doublet. Are both species LRRC25 in this precipitated immunoblot? The whole cell lysate that was analyzed in Figure 6D appears to be a single band. This requires further clarification.*

Response: To address this question, we had repeated this experiment using milk as antibody dilution buffer, and found that endogenous LRRC25 only showed a single band (**New Fig 2, related to Fig 3C in the manuscript**). The upper band in the doublet is an unspecific band, which was more obvious when we used BSA as antibody dilution buffer before. So we repeated our results using milk as antibody dilution buffer, and replaced the old images.

(Figure for referees not shown)

Comment 5. *The CARD domain appears to be the LRRC25-binding domain within RIG-I. For specificity experiment, it would be interesting to determine whether LRRC25 interacts with CARD of MDA5 and MAVS.*
Response: To address this question, we evaluated the interaction between LRRC25 and the CARDs of RIG-I, MDA5 and MAVS. We found that LRRC25 could interact with CARDs of RIG-I, MDA5, but not with MAVS (**New Fig 3, related to Fig EV3E in the manuscript**). Furthermore, we had already analyzed the interaction between LRRC25 and endogenous MAVS, and found that LRRC25 did not associate with endogenous MAVS by IC poly(I:C) treatment (Fig EV3D), suggesting that LRRC25 cannot interact with the CARDs of MAVS. Taken together, these results suggest that LRRC25 strongly interacts with the CARD domain of RLRs.

(Figure for referees not shown)

Comment 6. *The LRRC25 knockout cells had significantly higher level of RIG-I protein, without altered mRNA level? The knockout cells are very important reagent, the author could use more of these cells to validate more of the autophagic degradation of RIG-I.*
Response: RIG-I is an ISG gene, which is up-regulated by type I IFNs. As shown in Figure 2, knockout of LRRC25 could elevate the transcription of IFN-b and downstream ISGs. Thus, the mRNA level of RIG-I in LRRC25 knockout cells is higher than that in control cells upon viral infection. To exclude the potential influence of RIG-I level from mRNA level, we used CHX to block protein synthesis in WT and LRRC25 knockout cells, and found that the degradation of RIG-I is impaired upon IC poly(I:C) treatment (Fig EV5C). Furthermore, we used the bafilomycin A1 to demonstrate that the autophagic degradation of RIG-I is promoted by LRRC25 (Fig EV4E). More importantly, deficiency of critical autophagy regulators or cargo receptor, including Beclin-1, ATG5 and p62 could block the autophagic degradation of RIG-I promoted by LRRC25 (Fig 4I, 4J and 5E). Taken together, all these results support the conclusion that LRRC25 promote RIG-I for autophagic degradation.

Comment 7. *What do we know about the tissue specific expression of LRRC25, in relation to RIG-I expression, rather than cGAS for example.*
Response: It is hard to determine the correlation of LRRC25 and RIG-I expression in different tissues. Firstly, LRRC25 could only promote the degradation of RIG-I after viral infection, so their expression at mRNA level might not correlate (**New Fig 4**). If we check the tissue specific expression correlation between LRRC25 and RIG-I, we need to evaluate them at protein level after viral infection. This is difficult to conduct these experiemnts with human tissues. Furthermore, as the degradation of RIG-I promoted by LRRC25 is dependent on ISG15, and the functions of ISG15 in mice and human are different. In ISG15 KO mice, antiviral immune response is reduced or mildly affected (Lenschow et al, 2007; Morales et al, 2013), however, ISG15 deficiency in human increases viral resistance and type I IFN production (Speer et al, 2016; Zhang et al, 2015). Therefore, in this study, we mainly focused on the effect of LRRC25 on RIG-I at the cellular level.

(Figure for referees not shown)

Comment 8. *The discussion deserves to include a brief summary of the negative regulatory mechanisms controlling RIG-I in signaling output. These include phosphorylation, ubiquitination-mediated degradation and more recently described deamidation by viral factors.*
Response: As the reviewer suggested, we had added a brief summary of the negative regulatory mechanisms controlling RIG-I in the discussion as follows: “Several studies have reported that the activity and stability of RIG-I is regulated by multiple protein modifications. Proteasomal degradation of RIG-I could be initiated through K48-linked ubiquitination by several E3 ligases, including RNF125, CHIP and Siglec-G/c-Cbl (Arimoto et al, 2007; Chen et al, 2013; Zhao et al, 2016). In addition, the phosphorylation and deamidation also play important roles in the regulation of RIG-I activity. For instance, CKII and PKCa/b negatively regulate the activation of RIG-I through phosphorylating the CTD and CARDs domain of RIG-I, respectively, while PFAS could positively regulate RIG-I via deamidation (He et al, 2015; Maharaj et al, 2012; Sun et al, 2011). We and other also found that several Nod like receptors, including NLRC5 and NLRX1, could inhibit

type I IFN signaling through physically blocking the interaction between RIG-I and MAVS (Allen et al, 2011; Cui et al, 2010; Xia et al, 2011). ”

Thanks for the opportunity to improve the quality of our manuscript by incorporated these suggested changes.

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Zhao K, Zhang Q, Li X, Zhao D, Liu Y, Shen Q, Yang M, Wang C, Li N, Cao X (2016) Cytoplasmic STAT4 Promotes Antiviral Type I IFN Production by Blocking CHIP-Mediated Degradation of RIG-I. Journal of immunology (Baltimore, Md : 1950) 196: 1209-1217

3rd Editorial Decision 1 December 2017

Thank you for submitting your revised manuscript to us. I have run it by referee #3 again, who appreciates the introduced changes and now supports publication. I am thus pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE REPORT

Referee #3:

The authors have addressed my comments to a satisfactory level.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Rong-Fu Wang
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2017-96781R1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose the sample size according to the study of similar fields performed by other researchers. Normally, the data are representative of three independent experiments, n=3.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We tested for normality using SPSS (Statistical Product and Service Solutions).
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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