

Viral RNA polymerase as a SUMOylation decoy inhibits RNA quality control to promote potyvirus infection in plants



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have previously shown that the complex formed by Pelota and Hbs1, two components of “nonstop decay” and “no-go decay” RNA quality control pathways, is involved in restricting the accumulation of phytoviruses of the Potyviridae family, such as turnip mosaic virus (TuMV). They showed that Pelota is SUMOylated by the SUMO E2-conjugating enzyme SCE1, and that this modification is essential for the Pelota-HBS1 complex formation. Preventing Pelota SUMOylation blocks its restriction activity towards TuMV. In this follow-up study, Ge et al. reports that the RNA-dependent RNA polymerase (NIb) of TuMV acts as a decoy for the SUMOylation of Pelota. They show evidence that expression of NIb prevents the SUMOylation of Pelota in a dose-dependent manner, and that overexpression of NIb mimics the gene mis-regulation associated with a mutation in Pelota. They identified SUMO interacting motifs in TuMV NIb that are conserved in most potyvirids-encoded NIbs and showed that these NIbs also perturb Pelota-mediated restriction of viruses. Therefore, the authors conclude that they have identified a mechanism by which potyvirids counteract viral restriction by Pelota to promote infection.

Most data are convincing and well-presented throughout the manuscript. The text is clearly written and overall, this is a very interesting manuscript. I only have a few comments that the authors may want to address or answer, and I would like to draw the authors' attention to a few points where conclusions should either be toned down or fully demonstrated.

1- I think that the conclusions drawn from figure 1 are oversimplified and some points are not accurately described.

a. Line 128: in contradiction to the authors' statement, they do observe a significant increase of Pelota mRNA in rosette leaves upon TuMV infection (Fig 1a). Please rephrase or change “significant”. And correct typo line 129: Fig 1c should be 1a.

b. The main conclusion in Fig 1 is that there is a decreased sumoylation of Pelota upon TuMV infection. However, panels b, c and e show a decrease of Pelota in the input (especially in panel e). The authors should use biological replicates to quantify both levels of Pelota and sumoylated Pelota. I think this quantification is important because many conclusions in this manuscript are based on visual inspection of WB to compare protein levels.

c. Fig 1e. As there is a clear decrease of HBS1 but also of Pelota-YFP upon TuMV infection, it is therefore not surprising to detect less HBS1 in the IP. Accordingly, it cannot be concluded that “the decrease in Pelota SUMO modification showed a correlation with a reduced Hbs1 immunoprecipitation”. The reduced accumulation of HBS1 should be mentioned and considered by the authors in their interpretation of the data.

2- Fig 2b. Why do the levels of Pelota-YFP decrease in the input but increase in the IPs. Again, quantification using biological replicates would solve these issues.

3- Line 195: What is the meaning of “sufficiently blocked”?

4- Fig 5c. Why GST-Pelota is immunoprecipitated with YFP-MBP (left IP lanes)?

5- Fig 5e. The authors write “We further confirmed that the NIbsim3 mutation results in the loss of the ability of NIb to interact with SCE1 in the BiFC and Co-IP assays (Fig. 5d, e)”. I think that the data in Fig 5e is problematic. There is obviously much less NIbsim3 in the input as compared to NIbsim2. Therefore, it cannot be concluded that less NIbsim3 is IPed as compared to NIbsim2 (for which the signal is quite faint anyway). Again, quantification on biological replicates is required to support this conclusion.

6- Lines 338-340. “qRT-PCR analysis showed few viral genomic RNA in the newly developed leaves of plants infected with TuMV-NIbsim2 and TuMV-NIbsim3 (Fig. 5i), indicating that the mutated virus lost its capability to inhibit the Pelota-mediated RQC, resulting in ineffective infection to plants”. The authors have mutated a viral replicase. How can they be sure that the decrease in viral RNA titer is linked to inhibition of Pelota-mediated RQC and not a to a deleterious effect on viral replication? This possibility should be considered, especially because sim2, which has no impact on interaction between NIb and pelota similarly affects viral infection.

Reviewer #2 (Remarks to the Author):

Potyvirus RNA polymerase NIb is a multifunctional protein that interacts with several host proteins, including NPR1 in the nucleus to counter SA-mediated immune responses. Following on from the NIb functions at the nucleus, a series of studies have drawn a very interesting story around the SCE1-catalysed turnip mosaic virus (TuMV) NIb sumoylation. Sumoylated NIb is transferred to cytoplasm for viral replication. No-go decay (NGD) mediated by the Pelota-Hbs1 complex, which recognizes the G1-2A6-7 motif in the P3 cistron of potyviral RNA, negatively regulates infection. The current study indicates that NIb-SCE1 interaction competes with Pelota-SCE1 interaction and reduces Pelota sumoylation, which is a requirement for formation of Pelota-Hbs1 complex and NGD. Inhibition of NGD, a cellular RNA quality control (RQC) pathway, can therefore be regarded as a novel mechanism in the arms race between the virus and the host. The significance of the results is enhanced by the fact that both the site of sumoylation and the SCE1 interaction region in NIb are strongly conserved in the family Potyviridae. Interestingly, both the knock-out of Pelota and transgenic expression of NIb have significant and overlapping consequences on Arabidopsis transcriptome showing that this phenomenon is probably responsible for a significant part of the regulation of host gene expression during infection.

This work has been done to high standards. All conclusions have been drawn based on several corroborating and well-controlled experiments and mutually supporting results. I have few comments that I think would further clarify the manuscript:

1, As the mechanism of NDG involving Pelota-Hbs1 complex may not be familiar to all readers, I suggest adding more information to introduction. Then it would be clear later why involvement of Hbs1 is studied in the competitive Co-IP assays.

2. Sumoylated Pelota-Hbs1 complex functions in association to stalled ribosomes. Nlb-Pelota BiFC shows that Nlb and Pelota associates both in nucleus and cytoplasm. However, they don't interact physically, and the authors speculate that SCE1 is bridging the association. What is the cellular localization of SCE1 – can it be in cytoplasm linked to its substrates? Why Pelota and Nlb would be linked through SCE1? Could the authors elaborate the cell biology of the sumoylation of Pelota and formation of Pelota-Hbs1 complex.

3. Are all cellular Pelota functions blocked during TuMV infection? Can some information of this be deduced from the transcriptome analysis? Maybe the gene set enrichment analysis result in Supplementary Fig. 5d could be described in bigger detail.

4. The Pelota relevant transcriptional pattern is investigated in Supplementary Fig. 1. Since AGO2 is the main antiviral AGO for TuMV, it would be good to include the AGO2 regulation in pelota plants into the panel b.

Minor:

Row 126: TuMV infection for 14 days.

Row 128: May be this statement could be reworded: neither TuMV infection nor SA treatment significantly affected... I suggest this because Pelota expression increased slightly but in a statistically significant manner due to TuMV infection (Fig. 1a). OBS. in the text Fig. 1a is referred to as Fig. 1c on row 129.

Fig. 1b: Please, explain the sumoylated Pelota result in IP:GFP upper panel. Why there are two bands in the absence of TuMV infection? Why the sumoylated Pelota is smaller in the presence of TuMV infection than in healthy plant.

Row 151 and Fig. 1e: Hbs1 is reduced already in the input of Hbs1-Flag when TuMV concentration increases. Therefore, I wouldn't say that there is a correlation between Pelota SUMO modification and Hbs1 immunoprecipitation.

Row 178: Interestingly CI, VPg, NIa, Nlb and CP interact with SCE1 in Y2H assay. Maybe the authors could speculate why only Nlb is preventing Pelota's SUMOylation.

Fig. 4C is not described in the text.

The authors could explain the RFP-H2B marker in image legends describing confocal microscopy results.

Reviewer #3 (Remarks to the Author):

GE and co-workers address an interesting topic on the role of SUMO in plant defense against viruses. They have performed a large amount of work but, unfortunately, there are major conceptual issues that prevent the reader from making sense of some key experiments.

1. The SIM is a SUMO-Interacting Motif. Here is nice review on the subject,

Jara Lascorz, Joan Codina-Fabra, David Reverter, Jordi Torres-Rosell, SUMO-SIM interactions: From structure to biological functions, *Seminars in Cell & Developmental Biology*, 132, 2022, 193-202, <https://doi.org/10.1016/j.semcd.2021.11.007>.

As a SUMO interacting motif, and NOT an SCE1 interacting motif, it escapes from my understanding which is the rationale behind all the experiments trying to demonstrate the role of Nlb SIMs in SCE1 interactions. In addition, SIM1 and SIM2 sequences, as they are in an alpha-helix, do not have the structural requirements of a SIM, which is a beta-sheet.

2. Another conceptual problem, as I see it, is related to Nlb-Pelota interactions. In lines 232-237, the authors state:

“However, our Y2H and in vitro pull-down assays showed no direct interaction between Nlb and Pelota, indicating Nlb and Pelota do not physically interact. Since both Nlb and Pelota interact directly with SCE1 (Fig. 3d, e), we hypothesized that SCE1 might serve as a mediator in this interaction, bridging the interaction between Nlb and Pelota. These findings collectively imply the formation of an Nlb-Pelota complex in plant cells.”

If SCE1 bridges the interaction between Nlb and Pelota, it will be expected that SCE1 promotes the complex Nlb-SCE1-Pelota, but the results go in the opposite direction. This is summarized by the Fig4 legend: “Nlb disrupts the function of Pelota by competing with SCE1”

Accordingly, there is no relation between the capacity of Nlb to interact with SCE1 and the pathogenicity (Fig.5)

3. In addition, there are important controls missing:

- Fig. 1b. The proper negative control is YFP and not Col-0
- Fig. 1c. SUMO levels in the input samples.
- Fig. 1e. YFP negative control
- Fig. 2a. Input levels of SUMO, Pelota and the viral proteins

- Fig. 2b. SUMO input levels.
- Fig. 2e. It will be worthy to reevaluate the statistics analyses, mainly between control GFP and Pelota Nlb
- Fig. 3e. A SCE1-dose dependent assay with constant amounts of Nlb and Pelota will indicate if SCE1 promotes Nlb-Pelota complex.
- Fig. 4 c and d. Negative controls missing, GFP and MBP, respectively.

Reviewer #4 (Remarks to the Author):

The paper by Ge et al. describes a novel mechanism to suppress host-mediated degradation of viral RNAs by the potyviral protein Nib. Overall the manuscript is well presented and the data shown substantiates the claims.

Major comments

The authors demonstrate using Y2H (Fig 2 and S2) that SCE1 interacts with different viral proteins (C1, Nla-VpG, NLa-Pro and Nib), but that only Nlb appears to strongly suppress Pelota SUMOylation. Q: Did the authors investigate whether Nib inhibits the global SUMOylation response triggered e.g. by a heat shock in Arabidopsis? This test would demonstrate whether the inhibition of SCE1 activity by Nib is specific for Pelota only or generic inhibition. I missed this control.

The authors argue that about 856 genes are upregulated both in the pelota null mutant and OE:Nib. Did the authors look if the corresponding mRNAs of the genes carry any (un)known signature/feature that would make these transcripts a target of Pelota degradation? Is the effect seen for the majority of these DEGs therefore indirect? In other words, is the effect on gene expression linked to increased mRNA transcription or due to reduced mRNA turnover?

I missed in the Fig panels and/or methods how many times experiments were repeated independently. In particular, this holds for the IPs and in vitro protein expression/competition experiments. I strongly encourage the authors to upload all scans, images of the entire blots, films etc. for the various protein panels using FigShare or an alternative platform for the reader.

Likewise for Fig 1A and 1D. The authors show two samples in the bar? Is this the number of repeats in a single experiment? Or is this average of one experiment with multiple biological samples? This applies for the other RT-qPCR samples as well in other figures. Please clarify the total number of samples analyzed (including technical versus biological repeats in a single experiment). And how many times the experiment was repeated independently.

L142: Significant; Fig 1C does not show any quantification or statistical test meaning that the word 'significant' is not warranted. Please change the usage of this word across the entire manuscript. In fact, it is relevant to quantify, for example, the signal intensity ratios of the SUMOylated Pelota / Pelota-YFP in the IP. As the exposure times of the blots are different, it could be good to indicate this ratio (using the digital images of the ECL blots).

The experimental descriptions in the Materials and Methods is too brief in my opinion. (i) It is unclear to me e.g. in Fig 1F the authors mention a TuMV viral infected in combination with Pelota or Pelota/Nib 8 dpi. How is the Pelota and Nib expression performed? Using Agrobacterium? How many hours of expression etc. These details are missing in the caption and methods (or is known disconnected). (ii) I missed information on the plasmid design, backbones for the Agrobacterium transient expression system, the yeast two-hybrid system, plant transformation plasmids, in vitro expression etc. The reference in L532 is missing for the protoplast transfection. L585-586: how should I interpret the equal amount versus equal amounts. Please clarify the amounts used, protein concentrations, volumes etc. What is the composition of PBS in L594 etc. PBS can have different compositions, etc. The methods needs a strong revision to present the details needed to repeat the different experiments independently in another lab.

The statistical test in several figures appears to be a student-t test while the authors compare multiple samples. Why did the authors decide for a t-test rather than an ANOVA combined with an appropriate post-hoc test (Fig 2c, 2e, 2g etc). L167 states that the ns means no significant difference, but it is unclear which statistical test was used. Please add all of this information throughout the entire manuscript.

L234-237: The authors claim Nib and Pelota do directly interact but that NI and Pelota form a complex (Fig 3c and 3e). The formation of a complex linked by SCE1 is not critical to suppress the SUMOylation of Pelota when SCE1 would be sequestered by Nib. To substantiate this claim of a protein complex, the authors could use the Y3H system to test if SCE1 acts as bridge between the 2 proteins. And is this bridge function is impeded when the NI SIM3 motif is mutated. As an alternative test to Fig 5e.

The authors demonstrate that mutating the sim2 in Nib already results in loss of virulence. Do the authors have an explanation? I missed a proper discussion on this finding.

Minor comments:

Fig2A: I missed a control for the protein levels of the different viral proteins; Are they expressed to the same level for all viral proteins?

L228 vigorous?

L250 >Fig. 3b > Fig 3e

L189: What is the expression assay used? pUC transfections?

Point-by-Point Responses to Reviewers' Comments

Thanks to Dr. Wang for considering this manuscript. We also wish to thank four anonymous reviewers for providing us with constructive, insightful comments to improve the manuscript. Following the instruction in the Decision letter, we have carefully revised the manuscript essentially by addressing all the comments from the four reviewers and incorporating results from suggested experiments. All response text is in blue font, and the revised manuscript text shows major changes highlighted with tracked changes. A point-by-point response is provided for you to review. We hope that this revised version is acceptable for publication.

Reviewer #1 (Remarks to the Author):

The authors have previously shown that the complex formed by Pelota and Hbs1, two components of “nonstop decay” and “no-go decay” RNA quality control pathways, is involved in restricting the accumulation of phytoviruses of the Potyviridae family, such as turnip mosaic virus (TuMV). They showed that Pelota is SUMOylated by the SUMO E2-conjugating enzyme SCE1, and that this modification is essential for the Pelota-HBS1 complex formation. Preventing Pelota SUMOylation blocks its restriction activity towards TuMV. In this follow-up study, Ge et al. reports that the RNA-dependent RNA polymerase (NIb) of TuMV acts as a decoy for the SUMOylation of Pelota. They show evidence that expression of NIb prevents the SUMOylation of Pelota in a dose-dependent manner, and that overexpression of NIb mimics the gene mis-regulation associated with a mutation in Pelota. They identified SUMO interacting motifs in TuMV NIb that are conserved in most potyvirids-encoded NIbs and showed that these NIbs also perturb Pelota-mediated restriction of viruses. Therefore, the authors conclude that they have identified a mechanism by which potyvirids counteract viral restriction by Pelota to promote infection. Most data are convincing and well-presented throughout the manuscript. The text is clearly written and overall, this is a very interesting manuscript. I only have a few comments that the authors may want to address or answer, and I would like to draw the authors' attention to a few points where conclusions should either be toned down or fully demonstrated.

Response: We thank you for the very positive comments on the potential impact of this work, and for providing us with the following suggestions to improve the manuscript.

1- I think that the conclusions drawn from figure 1 are oversimplified and some points are not accurately described.

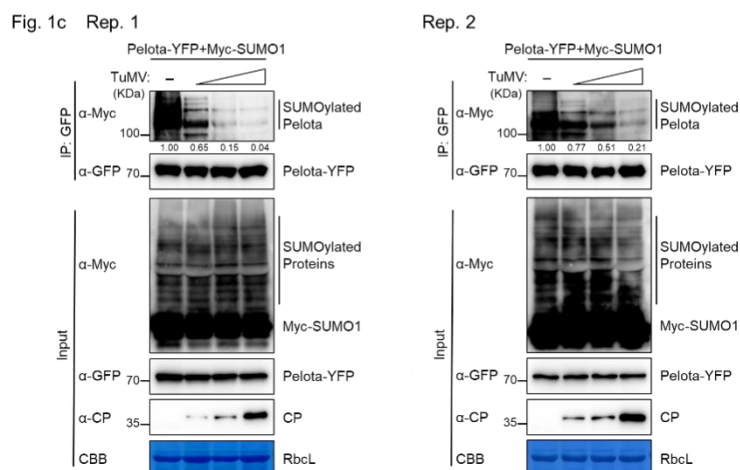
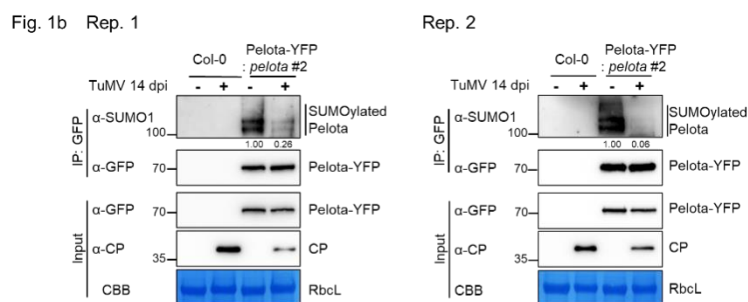
a. Line 128: in contradiction to the authors' statement, they do observe a significant increase of Pelota mRNA in rosette leaves upon TuMV infection (Fig 1a). Please rephrase or change “significant”. And correct typo line 129: Fig 1c should be 1a.

Response: Thank you for this suggestion. Indeed, TuMV infection resulted in a slight upregulation of *Pelota* mRNA expression, approximately 10%. *Pelota* expression increased slightly but in a statistically significant manner due to TuMV infection in rosette leaves. Therefore, we rephrased this sentence: “However, quantitative RT-PCR (qRT-PCR) revealed a slight upregulation of *Pelota* after wounding (0.5 h) and cold treatments,

and TuMV infected rosette leaves (Fig. 1a, Supplementary Fig. 1a). And we have corrected this Fig reference.

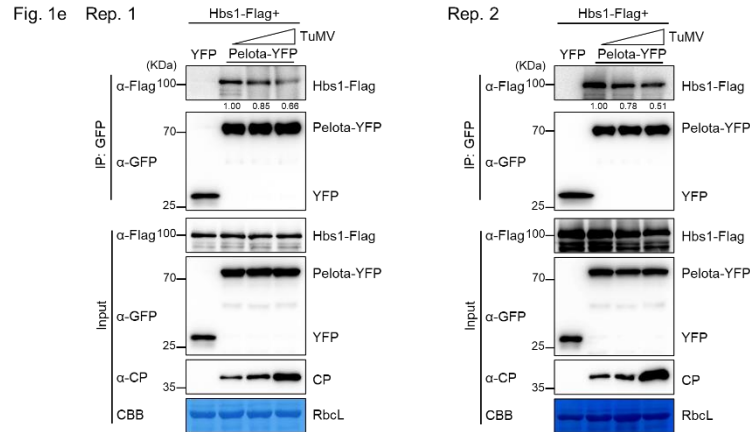
b. The main conclusion in Fig 1 is that there is a decreased sumoylation of Pelota upon TuMV infection. However, panels b, c and e show a decrease of Pelota in the input (especially in panel e). The authors should use biological replicates to quantify both levels of Pelota and sumoylated Pelota. I think this quantification is important because many conclusions in this manuscript are based on visual inspection of WB to compare protein levels.

Response: Thank you for this suggestion. We observed that TuMV infection reduced the SUMOylation of Pelota, thereby leading to the degradation of the Pelota-Hbs1 RQC complex. We unexpectedly found that MG132, a proteasome inhibitor, can rescue these degradation events. We speculate that ubiquitination coupled with SUMOylation plays a critical role in the RNA quality control system. However, we have not identified the molecular mechanistic connection between them so far and this could be explored in the near future. To solve this concern, we performed another two independent biological replicates of these experiments under the MG132 treatment and provided quantitative data according to your suggestion in the revised manuscript.



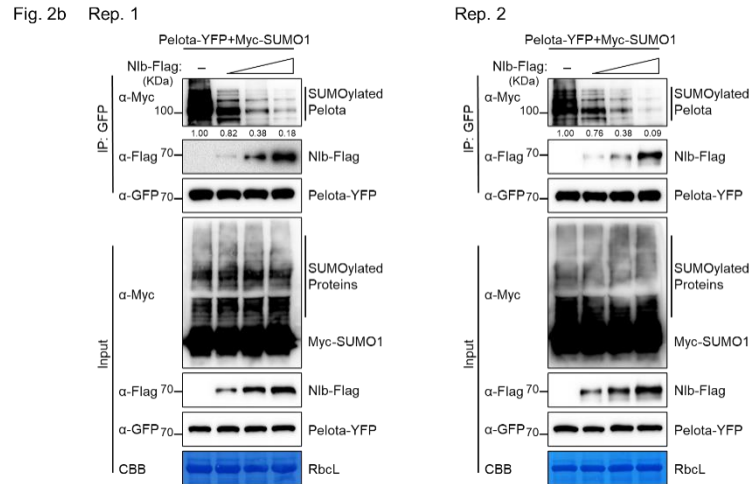
c. Fig 1e. As there is a clear decrease of HBS1 but also of Pelota-YFP upon TuMV infection, it is therefore not surprising to detect less HBS1 in the IP. Accordingly, it cannot be concluded that “the decrease in Pelota SUMO modification showed a correlation with a reduced Hbs1 immunoprecipitation”. The reduced accumulation of HBS1 should be mentioned and considered by the authors in their interpretation of the data.

Response: Thank you for this suggestion. Similar observations were mentioned as above. We performed another two independent biological replicates of these experiments under the MG132 treatment. The presence of MG132 prevented the degradation of Hbs1 by the ubiquitin 26S proteasome system, and in this case, we can still observe the disruption of the Pelota-Hbs1 interaction during TuMV infection, ensuring the validity of this conclusion. The quantitative data of the WB bands have been added in the figures.



2- Fig 2b. Why do the levels of Pelota-YFP decrease in the input but increase in the IPs. Again, quantification using biological replicates would solve these issues.

Response: Thank you for pointing this out. Similar observations were mentioned as above. We performed two additional independent biological replicates of WB experiments under the MG132 treatment. As seen in the following results, the presence of MG132 prevented the degradation of Pelota by the ubiquitin 26S proteasome system, and in this case, we still observed the decrease of the SUMOylated Pelota in the presence of Nib, ensuring the reliability of this conclusion. The quantitative data of the WB bands have been added in the figures.



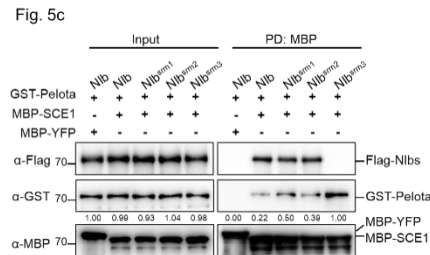
3- Line 195: What is the meaning of “sufficiently blocked”?

Response: Thank you for pointing this out. To avoid ambiguity and make our results more rigorous, we have changed “sufficiently blocked” into “significantly diminished”.

Our studies show that transient expression of Pelota in plants inhibited virus infection. However, when NIb was co-expressed with Pelota, this inhibition of Pelota on viral infection was significantly diminished.

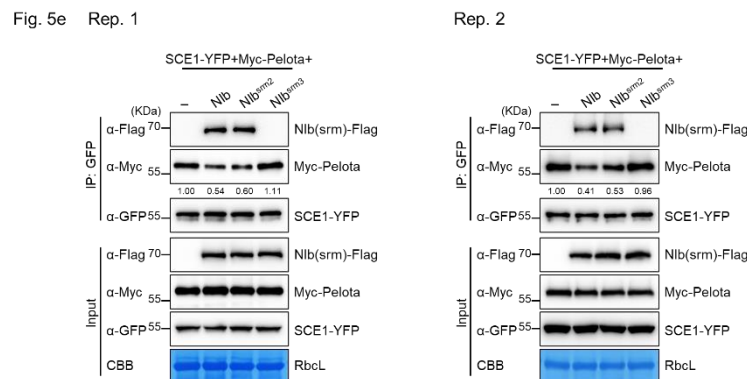
4- Fig 5c. Why GST-Pelota is immunoprecipitated with YFP-MBP (left IP lanes)?

Response: Thank you for pointing this out. We have corrected this in the revised manuscript.



5- Fig 5e. The authors write “We further confirmed that the Nlbsim3 mutation results in the loss of the ability of NIb to interact with SCE1 in the BiFC and Co-IP assays (Fig. 5d, e)”. I think that the data in Fig 5e is problematic. There is obviously much less Nlbsim3 in the input as compared to Nlbsim2. Therefore, it cannot be concluded that less Nlbsim3 is IPed as compared to Nlbsim2 (for which the signal is quite faint anyway). Again, quantification on biological replicates is required to support this conclusion.

Response: Thank you for pointing this out. In fact, Nlb^{srn3} (we changed SIM to SRM, SUMOylation-related motif, to avoid misunderstanding) has lower protein stability when detected by WB at the same infiltrated OD₆₀₀ (which reinforces our conjecture that ubiquitination may cross talk with SUMOylation in the protein stability of Pelota, Hbs1, and NIb). To exclude the effect derived from protein accumulation levels, we increased the Nlb^{srn3} protein accumulation by use of a higher density of Agrobacterium cultures carrying the Nlb^{srn3} construct (a higher OD₆₀₀), to make it accumulate to a similar level with wild-type NIb. This experiment has been repeated twice and a similar conclusion has been obtained. The quantitative data of the WB bands have been added in the figures.



6- Lines 338-340. “qRT-PCR analysis showed few viral genomic RNA in the newly developed leaves of plants infected with TuMV-Nlbsim2 and TuMV-Nlbsim3 (Fig. 5i), indicating that the mutated virus lost its capability to inhibit the Pelota-mediated RQC,

resulting in ineffective infection to plants". The authors have mutated a viral replicase. How can they be sure that the decrease in viral RNA titer is linked to inhibition of Pelota-mediated RQC and not a deleterious effect on viral replication? This possibility should be considered, especially because sim2, which has no impact on interaction between Nlb and pelota similarly affects viral infection.

Response: We agree with your comments. In this study, we identified the Nlb SRM3 motif is responsible for interacting with SCE1, mediating the Pelota inhibition and SUMOylation. Therefore, the Nlb^{srn3} mutation not only loses the ability to disrupt the Pelota-SCE1 interaction, but also interferes with its own SUMOylation, leading to a decrease in viral pathogenicity. However, we cannot rule out the potential impact of these mutations on replicase activity. We have now discussed the potential effects of the Nlb^{srn2} and Nlb^{srn3} mutants on viral replication in the revised manuscript.

Reviewer #2 (Remarks to the Author):

Potyvirus RNA polymerase Nlb is a multifunctional protein that interacts with several host proteins, including NPR1 in the nucleus to counter SA-mediated immune responses. Following on from the Nlb functions at the nucleus, a series of studies have drawn a very interesting story around the SCE1-catalysed turnip mosaic virus (TuMV) Nlb sumoylation. Sumoylated Nlb is transferred to cytoplasm for viral replication. No-go decay (NGD) mediated by the Pelota-Hbs1 complex, which recognizes the G1-2A6-7 motif in the P3 cistron of potyviral RNA, negatively regulates infection. The current study indicates that Nlb-SCE1 interaction competes with Pelota-SCE1 interaction and reduces Pelota sumoylation, which is a requirement for formation of Pelota-Hbs1 complex and NGD. Inhibition of NGD, a cellular RNA quality control (RQC) pathway, can therefore be regarded as a novel mechanism in the arms race between the virus and the host. The significance of the results is enhanced by the fact that both the site of sumoylation and the SCE1 interaction region in Nlb are strongly conserved in the family Potyviridae. Interestingly, both the knock-out of Pelota and transgenic expression of Nlb have significant and overlapping consequences on Arabidopsis transcriptome showing that this phenomenon is probably responsible for a significant part of the regulation of host gene expression during infection.

This work has been done to high standards. All conclusions have been drawn based on several corroborating and well-controlled experiments and mutually supporting results. I have few comments that I think would further clarify the manuscript:

Response: Thank you for your very positive comments on our work and also for suggestions to strengthen this manuscript.

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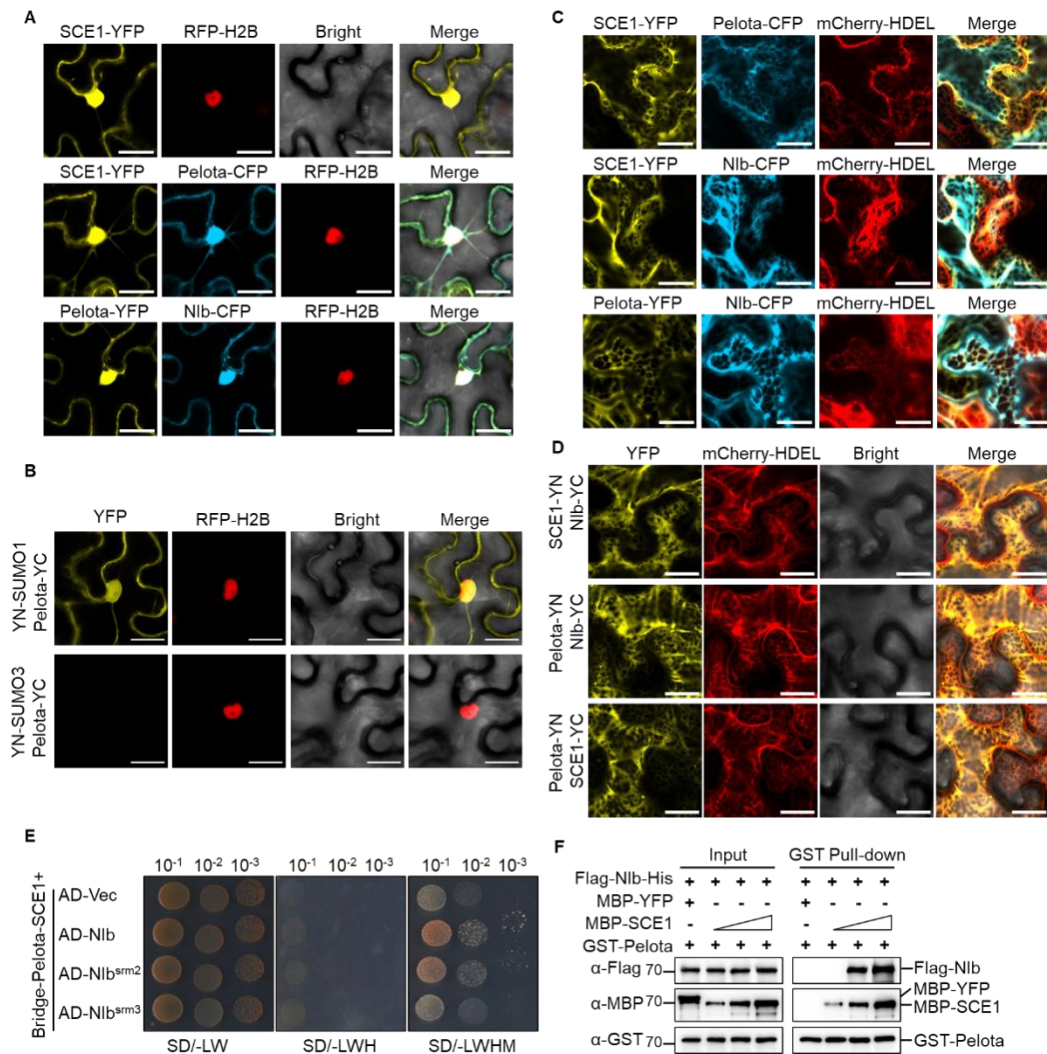
Response: Thank you for pointing this out. We have added additional details about the recruitment of Hbs1 by SUMO1-modified Pelota and highlighted its importance in NGD in the revised manuscript.

2. Sumoylated Pelota-Hbs1 complex functions in association to stalled ribosomes. Nlb-Pelota BiFC shows that Nlb and Pelota associates both in nucleus and cytoplasm. However, they don't interact physically, and the authors speculate that SCE1 is bridging the association. What is the cellular localization of SCE1 – can it be in cytoplasm linked to its substrates? Why Pelota and Nlb would be linked through SCE1? Could the authors elaborate the cell biology of the sumoylation of Pelota and formation of Pelota-Hbs1 complex.

Response: Thank you for these comments. SCE1, Nlb, and Pelota are localized in the nucleus and cytoplasm (this study), but Hbs1 and Hbs1-Pelota interaction complex are only localized in the cytoplasm (this result has been published previously). In normal conditions, Pelota interacts with SCE1 in the nucleus and cytoplasm for SUMOylation, and then the SUMO1-modified Pelota binds to Hbs1 in the cytoplasm to initiate NGD. During virus infection, the viral Nlb also needs SUMOylation by interacting with SCE1 in the nucleus and cytoplasm for its functionality, therefore the virus-expressed Nlb would outcompete Pelota for binding with SCE1. This leads to the indirect interaction of Nlb and Pelota in the nucleus and cytoplasm, and disruption of Pelota's SUMOylation, thereby inhibiting the formation of Pelota-Hbs1 complex.

To strengthen this conclusion, we have also conducted a number of additional experiments to better support the proposed model in this study:

- We provided the subcellular localization of SCE1 showing that SCE1 is localized in the nucleus and cytoplasm. We also examined the co-localization of SCE1-YFP with Pelota-CFP in RFP-H2B (nucleus localization marker) transgene plants, as well as co-localization of SCE1-CFP with Pelota-YFP in RFP-H2B transgene plants (The following Fig A).
- We conducted another experiment using N-terminally tagged YFP-N-SUMO1 co-expressed with Pelota-YFP-C (YFP-N-SUMO3 was used as control). This allowed us to observe the SUMO1-modified Pelota in cells. By co-expressing Pelota, SCE1, and Nlb in cells, we can observe the co-localization of the three components in the nucleus and cytoplasm endoplasmic reticulum (The following Fig B-D).
- We provided a Y3H analysis that demonstrated the interaction between Pelota and Nlb using SCE1 as a bridge. In addition, GST-Pelota failing to pull down Nlb without the presence of SCE1 was also provided in the revised manuscript (The following Fig E-F).



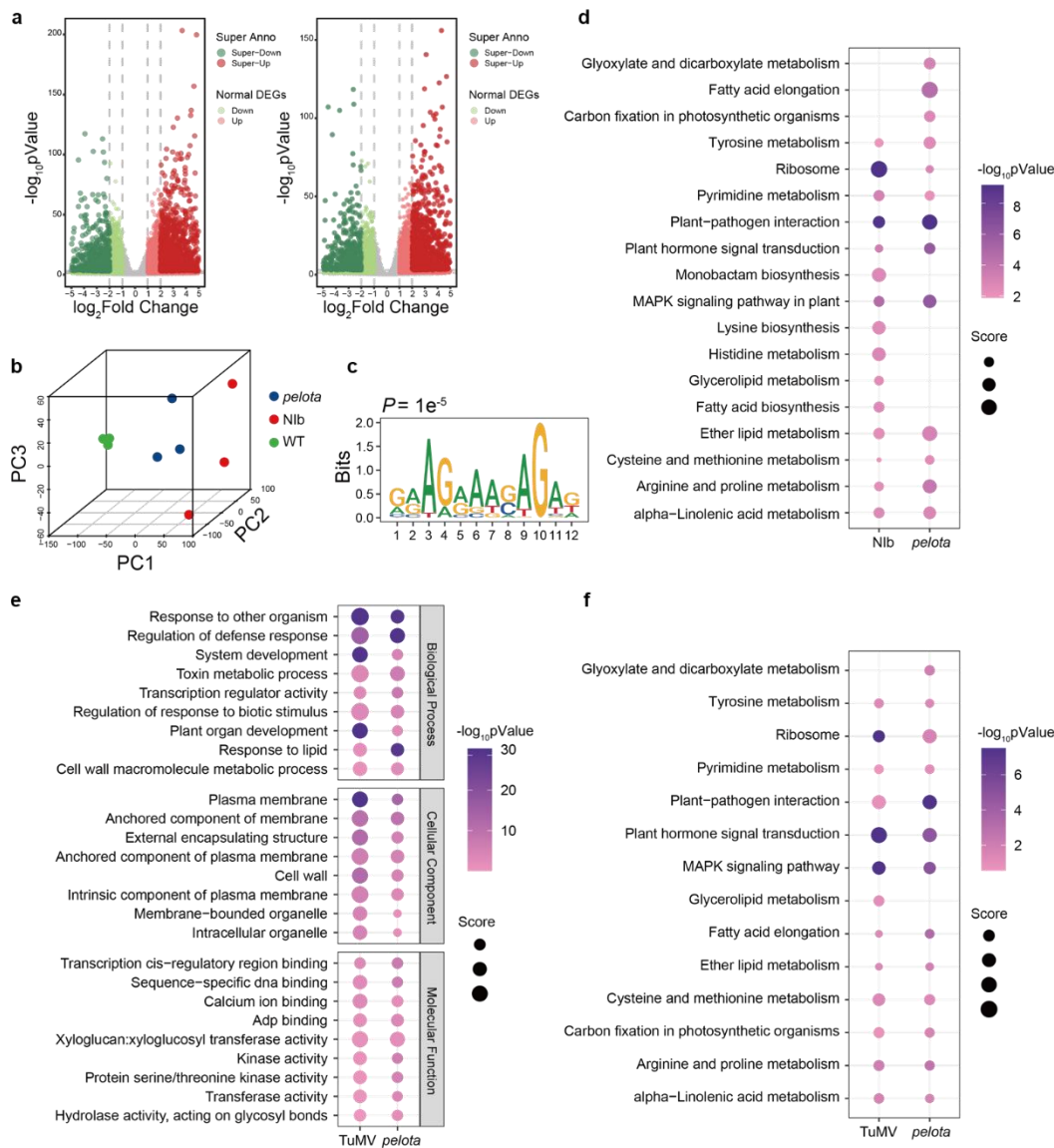
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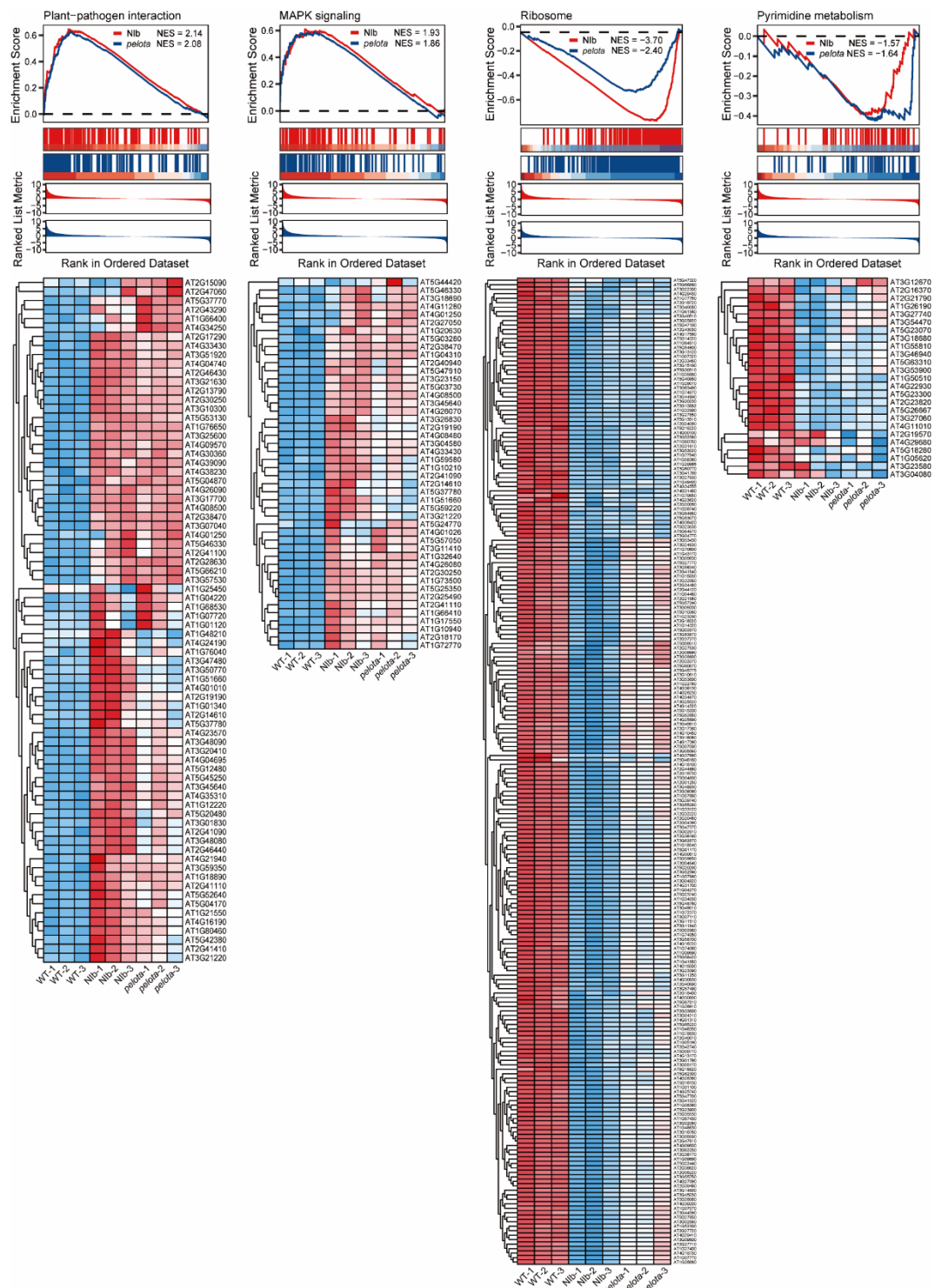
Response: Thank you for these comments. Based on our transcriptome analysis, it is clear that TuMV infection leads to an impact on almost all Pelota regulatory genes.

As suggested, we provided additional RNAseq analyses by incorporating the transcriptome data of TuMV-infected Arabidopsis plants. We observed that differentially expressed genes (DEGs) in *pelota* mutant were similar to those in TuMV infection. GO and KEGG functional enrichment analysis showed that TuMV infection highly covers the Pelota-regulated gene set (New Supplementary Fig. 6e, f).

The GSEA (Gene Set Enrichment Analysis) results demonstrate a high degree of consistency in the pathways activated or suppressed by Nlb and *pelota*. Upregulated genes showed significant enrichment in Nlb and *pelota* in the Plant-pathogen interaction and MAPK-related pathways ($p < 0.0001$, $FDR < 0.0001$, $NES > 1.8$). Conversely, both Nlb and *pelota* significantly repressed the Ribosome and Pyrimidine metabolism pathways ($p < 0.0001$, $FDR < 0.0001$, $NES < -1.5$). We now provide heatmaps of these genes in Col-0, Nlb, and *pelota* to support this result (New Supplementary Fig. 7). Further details of the

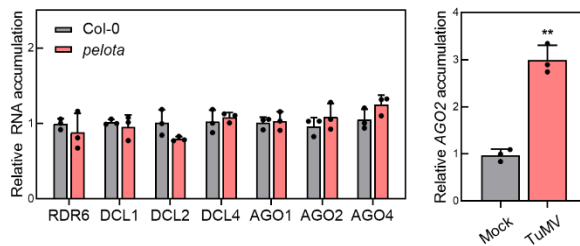
manuscript describing the GSEA and the new GO, KEGG analysis are provided in this revision.





4. The Pelota relevant transcriptional pattern is investigated in Supplementary Fig. 1. Since AGO2 is the main antiviral AGO for TuMV, it would be good to include the AGO2 regulation in pelota plants into the panel b.

Response: Thank you for this suggestion. We tested the expression level of AGO2 mRNA in *pelota* mutant, and found that the mRNA accumulation of AGO2 was not correlated with Pelota's expression, similar to other RNAi pathway genes (Supplementary Fig. 1b). As suggested, AGO2 is the main antiviral AGO for TuMV, and we also confirm that TuMV infection could upregulate the AGO2 expression level.



Minor:

Row 126: TuMV infection for 14 days.

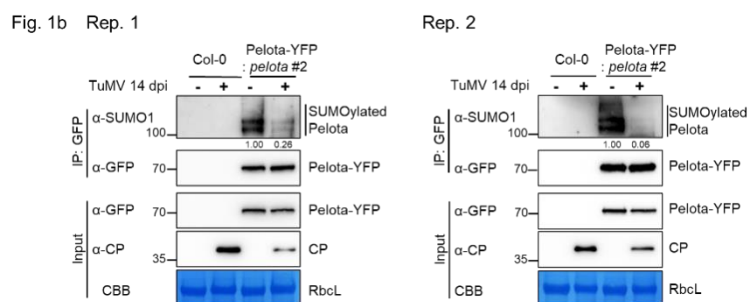
Response: Done as suggested.

Row 128: May be this statement could be reworded: neither TuMV infection nor SA treatment significantly affected... I suggest this because Pelota expression increased slightly but in a statistically significant manner due to TuMV infection. OBS. in the text Fig. 1a is referred to as Fig. 1c on row 129.

Response: Thanks for this suggestion. We have considered the use of the word "revealed a slight upregulation" (Fig. 1a, Supplementary Fig. 1a). And we have corrected this Fig reference.

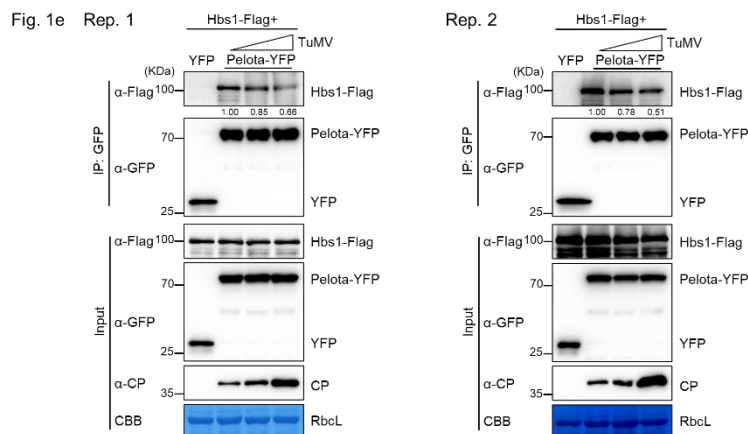
Fig. 1b: Please, explain the sumoylated Pelota result in IP: GFP upper panel. Why there are two bands in the absence of TuMV infection? Why the sumoylated Pelota is smaller in the presence of TuMV infection than in healthy plant.

Response: Thank you for pointing this out. The SUMOylated Pelota signal was too weak in TuMV-infected plants, leading to the ambiguous result. We now provide another two independent biological replicates of these experiments in the revised version.



Row 151 and Fig. 1e: Hbs1 is reduced already in the input of Hbs1-Flag when TuMV concentration increases. Therefore, I wouldn't say that there is a correlation between Pelota SUMO modification and Hbs1 immunoprecipitation.

Response: Thank you for pointing this out. We noticed that TuMV infection induced Hbs1 degradation in input samples together with Pelota. We also found that MG132, a proteasome inhibitor, can rescue these degradation events. In the revised manuscript, we performed another two independent biological replicates of these experiments under the MG132 treatment and provided quantitative data, to exclude the effect derived from protein accumulation variations.



Row 178: Interestingly CI, VPg, NIa, NIb and CP interact with SCE1 in Y2H assay. Maybe the authors could speculate why only NIb is preventing Pelota's SUMOylation.

Response: Thank you for this comment. Indeed, several viral proteins could interact with SCE1 and we confirmed only NIb inhibited the Pelota SUMOylation (Fig. 2a). As to why only NIb is able to prevent Pelota's SUMOylation, we have three hypotheses:

(1) These viral proteins interacting with SCE1 might be at different motifs on SCE1 from NIb, thus they have a fewer effect on the Pelota-SCE1 interaction and the Pelota's SUMOylation;

(2) The specific spatial conformation of NIb protein may be an obstacle to the Pelota-SCE1 interaction;

(3) NIb, SCE1, and Pelota interacted with each other and exhibited similar subcellular localizations in the nucleus, cytoplasm, and endoplasmic reticulum. SUMO1-modified Pelota was also found in these cellular regions. The similar distribution of subcellular localization could explain why the interaction between Pelota and SCE1 is competed by NIb. However, CI, VPg, NIa, and CP displayed different subcellular localizations.

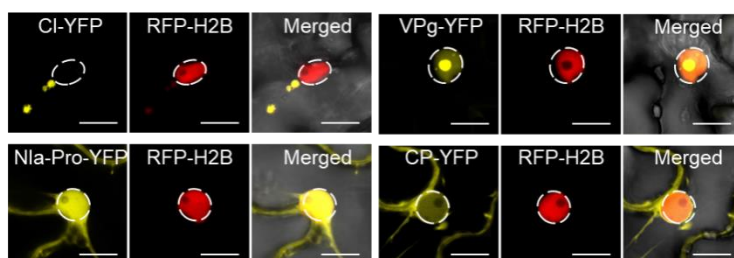


Fig. 4C is not described in the text. The authors could explain the RFP-H2B marker in image legends describing confocal microscopy results.

Response: Thank you for pointing this out. Fig 4c has been described as suggested. Transgenic RFP-H2B *N. benthamiana* was a gift from Michael M. Goodin (University of Kentucky, USA). We have added the RFP-H2B *N. benthamiana* information in the manuscript: a nuclear marker, red fluorescent protein fused to histone 2B (Fig. 3a, 5d, 6b, Supplementary Fig. 4a, 8a).

Reviewer #3 (Remarks to the Author):

GE and co-workers address an interesting topic on the role of SUMO in plant defense against viruses. They have performed a large amount of work but, unfortunately, there are major conceptual issues that prevent the reader from making sense of some key experiments.

Response: We thank the reviewer for your opinion and critical comments. We appreciate your time and effort, and thank you for helping us to clarify our presentation.

1. The SIM is a SUMO-Interacting Motif. Here is nice review on the subject, Jara Lascorz, Joan Codina-Fabra, David Reverter, Jordi Torres-Rosell, SUMO-SIM interactions: From structure to biological functions, *Seminars in Cell & Developmental Biology*, 132, 2022, 193-202, <https://doi.org/10.1016/j.semcdb.2021.11.007>.

As a SUMO interacting motif, and NOT an SCE1 interacting motif, it escapes from my understanding which is the rationale behind all the experiments trying to demonstrate the role of Nlb SIMs in SCE1 interactions. In addition, SIM1 and SIM2 sequences, as they are in an alpha-helix, do not have the structural requirements of a SIM, which is a beta-sheet.

Response: Thank you for providing us these valuable comments, suggestions and the nice review of SUMO-SIM contacts for citation. Indeed, the SIM domain means SUMO interacting motif rather than SCE1 interacting motif.

To make our results more precise, we have changed our previous ambiguous “SIM” motifs into “SUMOylation-related motif (SRM)” to match our results and conclusions in this study. We found that TuMV infection inhibits Pelota’s SUMOylation, subsequently identified Nlb, a TuMV-encoded protein, responsible for this inhibition. Therefore, we explored the potential connection between Nlb and Pelota in the process of SUMO modification. Both Nlb and Pelota can interact with the SUMO E2 SCE1, leading us to investigate the relationships between the biological functions and the interactions among these three proteins. Our lab stored a variety of Nlb mutants including several previously identified SIM mutants. We then screened the interaction between SCE1 with these Nlb mutants and found the Nlb^{srn3} lost this ability and this conclusion was further confirmed through various experiments. Actually, we know that SIM means SUMO interacting motif, however, SRM3 (LLSV) displays a SIM-like characteristic, which is [VIL]-x-[VIL]-[VIL] or [VIL]-[VIL]-x-[VIL]. Therefore, SIM-like motifs might also contribute to the interaction with SCE1, representing a novel finding in our study.

The SIM2 is crucial for Nlb SUMO modification and viral virulence, which has been reported previously (Ref. Cheng et al., 2017, *Plant Cell*). We have highlighted this previous work in the manuscript. Nlb’ protein structure was predicted by the AlphaFold2 with high confidence. We admit further analysis is needed to clarify this predicted structure in response to your concerns, which we believe is beyond the scope of this study.

We are appreciated for your advice and agree with this structure prediction could be misunderstanding. Thus, we have removed it from Fig.6 in the revised manuscript and all SIM motifs have been changed into SRM motifs.

2. Another conceptual problem, as I see it, is related to Nlb-Pelota interactions. In lines 232-237, the authors state:

“However, our Y2H and *in vitro* pull-down assays showed no direct interaction between Nlb and Pelota, indicating Nlb and Pelota do not physically interact. Since both Nlb and Pelota interact directly with SCE1(Fig. 3d, e), we hypothesized that SCE1 might serve as a mediator in this interaction, bridging the interaction between Nlb and Pelota. These findings collectively imply the formation of an Nlb-Pelota complex in plant cells.”

If SCE1 bridges the interaction between Nlb and Pelota, it will be expected that SCE1 promotes the complex Nlb-SCE1-Pelota, but the results go in the opposite direction. This is summarized by the Fig4 legend: “Nlb disrupts the function of Pelota by competing with SCE1”. Accordingly, there is no relation between the capacity of Nlb to interact with SCE1 and the pathogenicity (Fig.5)

Response: Thank you for your comments. We provided additional *in vivo* pull-down analysis and Y3H analysis. GST-Pelota failing to pull down Nlb without the presence of SCE1 was provided in the revised manuscript (Following Fig. A). Y3H analysis also detected the interaction between Pelota and Nlb using SCE1 as a bridge (Following Fig. B). These results could support the existence of Nlb-SCE1-Pelota complex.

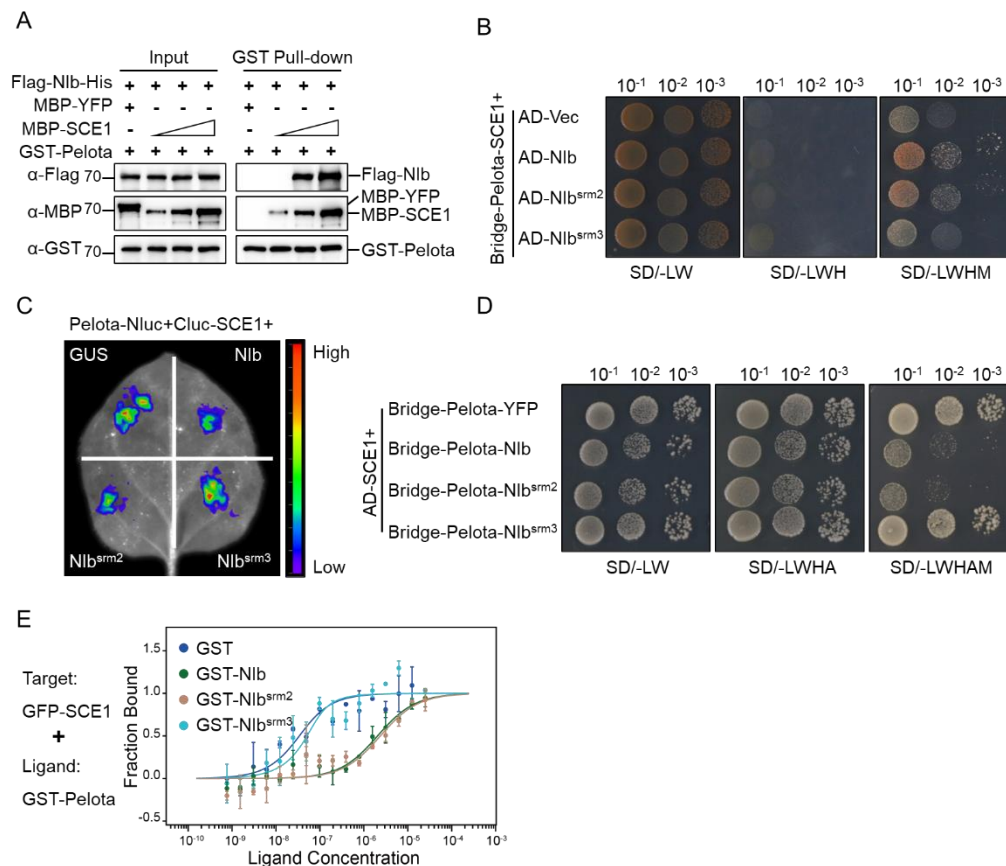
We agree that it is reasonable to assume that SCE1 promotes the Nlb-Pelota complex formation, as SCE1 can interact with both Pelota and Nlb *in vivo* and *in vitro*. However, our experiments revealed that Nlb inhibits the interaction between SCE1 and Pelota, suggesting Nlb competing with Pelota for binding with SCE1. This inhibition was abolished when the interaction motif between Nlb and SCE1 was mutated (BiFC, competitive Co-IP, competitive Pull-down experiments were provided, in Fig 5, in the revised version).

To directly address this point, we also conducted additional experiments. We expressed Nlb, Nlb^{srm2}, and Nlb^{srm3} in the Pelota-SCE1 LCI experiment. It was observed that Nlb and Nlb^{srm2}, but not Nlb^{srm3}, could obviously disrupt the fluorescence intensity of the interaction signal generated from Pelota and SCE1 (Following Fig. C). Y3H using Nlb, Nlb^{srm2}, and Nlb^{srm3} as the bridge also showed a similar result (Following Fig. D).

We provided additional *in vivo* evidence using Microscale Thermophoresis (MST) to analyze the Pelota-SCE1 interaction. The interaction strength between Pelota and SCE1 was at the nanomolar (nM) level. However, upon the addition of Nlb to the incubation system, the interaction strength decreases to the micromolar (uM) level (Following Fig. E). This is in our view a very strong argument that support our claims.

In this study, we identified the Nlb SRM3 motif is responsible for interacting with SCE1, mediating the Pelota inhibition and SUMOylation. Therefore, the Nlb srm3 mutation not only loses the ability to disrupt the Pelota-SCE1 interaction, but also interferes with its own SUMOylation, leading to a decrease in viral pathogenicity. We believe these data could better support our model, that viral infection results in increased

Nlb accumulation, leading to Nlb competing with Pelota for binding to SCE1 for SUMOylation. The data have been added in the revised manuscript.



In addition, our previous data showed that Nlb indeed required SCE1 for its SUMOylation and viral infectivity (Xiong et al., 2015). Therefore, it is reasonable to speculate that Nlb disrupts the complex SCE1-Pelota because of the limited amount of SCE1 in plants. So, the Nlb-SCE1 interaction inhibited the SCE1-Pelota interaction and decreased the Pelota's SUMOylation.

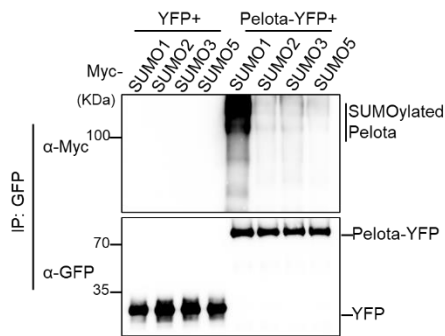
Xiong R, Wang A. SCE1, the SUMO-conjugating enzyme in plants that interacts with Nlb, the RNA-dependent RNA polymerase of Turnip mosaic virus, is required for viral infection. J Virol. 2013 Apr;87(8):4704-15

3. In addition, there are important controls missing:

- Fig. 1b. The proper negative control is YFP and not Col-0

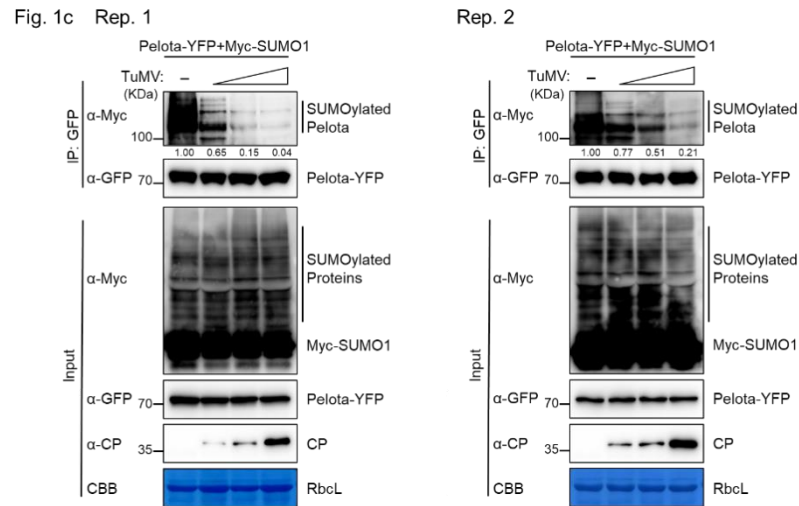
Response: Thank you for this suggestion. We agree that YFP plants are ideal controls for this analysis. However, we do not have stable YFP genetic lines that could be used in this study. It would take several months to obtain a YFP transgene plant. To test whether there is a different result when YFP is in or not, we expressed YFP or Pelota-YFP together with four Myc-tagged SUMO paralogs (SUMO1, SUMO2, SUMO3, or SUMO5) in *N. benthamiana* plants, respectively. As seen in the following figure, when YFP or Pelota-YFP conjugates immunoprecipitated using anti-GFP beads and immunoblotted with anti-Myc antibodies, only SUMO1-modified Pelota-YFP could be detected. Thus, we

think the YFP-tag has no effect on this conclusion, and we hope this result may solve your concern.



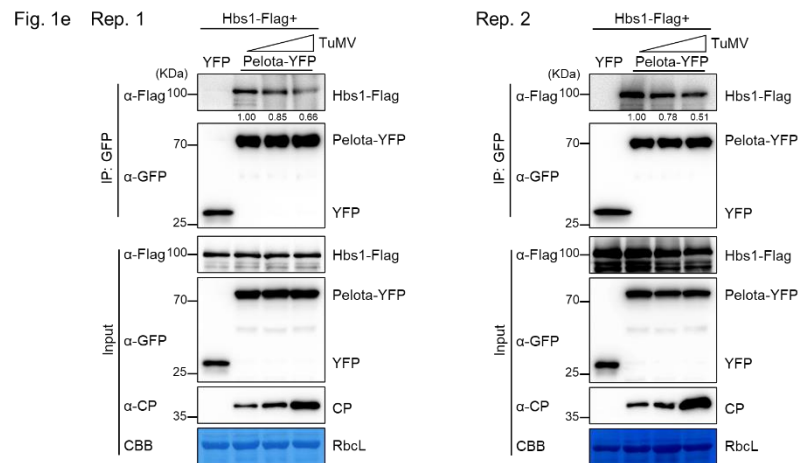
- Fig. 1c. SUMO levels in the input samples.

Response: We performed another two independent replicates of Fig. 1c, and provided the SUMO levels in the input samples.



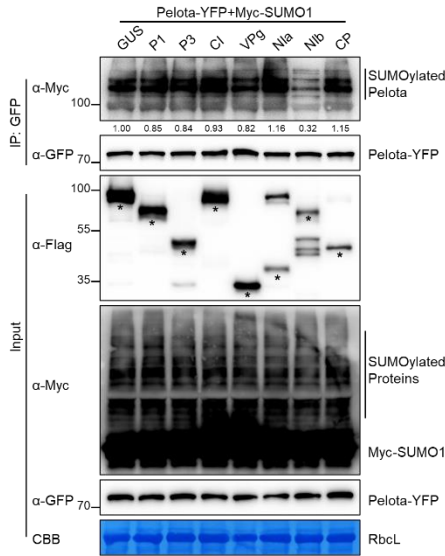
- Fig. 1e. YFP negative control

Response: We performed another two independent replicates of Fig. 1e, and added the YFP as a negative control.



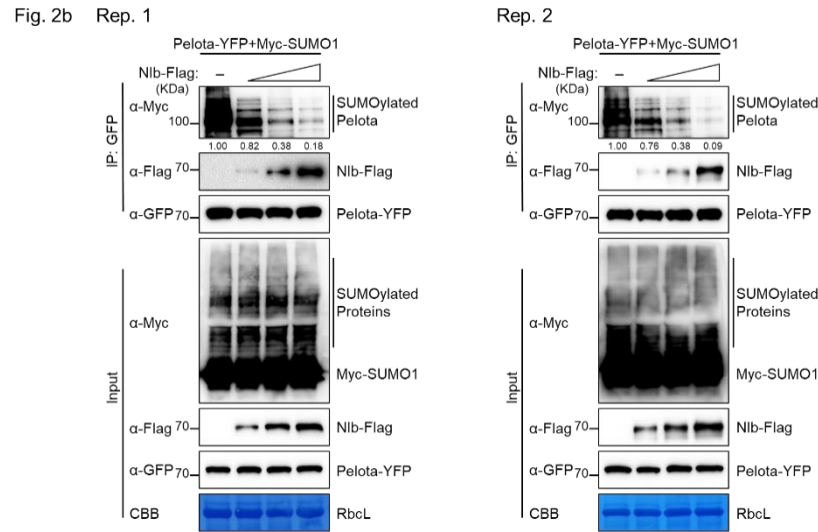
- Fig. 2a. Input levels of SUMO, Pelota and the viral proteins

Response: We have provided new Fig. 2a, and added the SUMO levels and viral proteins in the input samples.



- Fig. 2b. SUMO input levels.

Response: We performed another two independent replicates of Fig. 2b, and added the SUMO levels in the input samples.



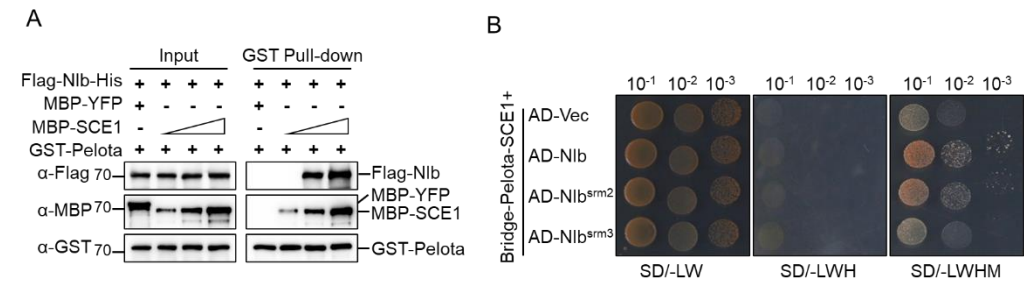
- Fig. 2e. It will be worthy to reevaluate the statistics analyses, mainly between control GFP and Pelota Nib

Response: Thank you for this suggestion. We reevaluated the statistics analyses of these data and obtained similar results.

We have provided the detailed descriptions on how these data were processed in the revised manuscript. Multiple comparisons were performed using GraphPad Prism with one-way ANOVA followed by Dunnett's or Tukey multiple comparisons test. Pairwise comparisons were performed by two-tailed *Student's t* test.

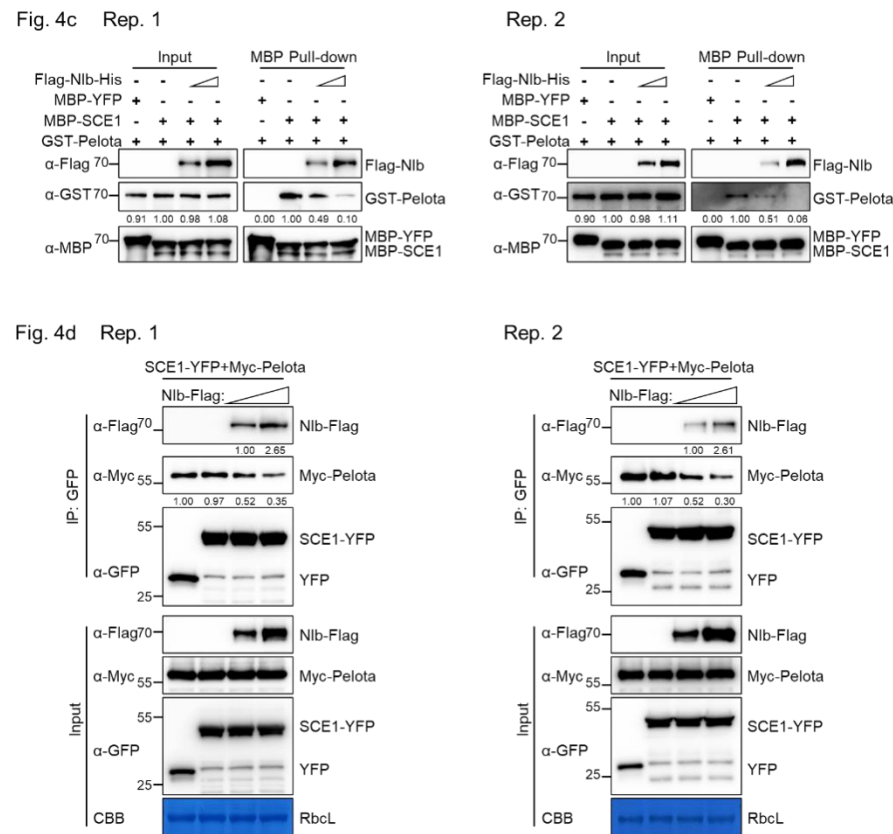
- Fig. 3e. A SCE1-dose dependent assay with constant amounts of Nlb and Pelota will indicate if SCE1 promotes Nlb-Pelota complex.

Response: Thank you for this suggestion. We performed additional experiments, which showed that SCE1 bridged the Pelota and Nlb, and the increased SCE1 promoted Nlb-Pelota complex (Following Fig. A-B).



- Fig. 4 c and d. Negative controls missing, GFP and MBP, respectively.

Response: Thank you for pointing this out. Additional experiments with negative controls (See the following figure) have been added in this result in the revised manuscript.



Reviewer #4 (Remarks to the Author):

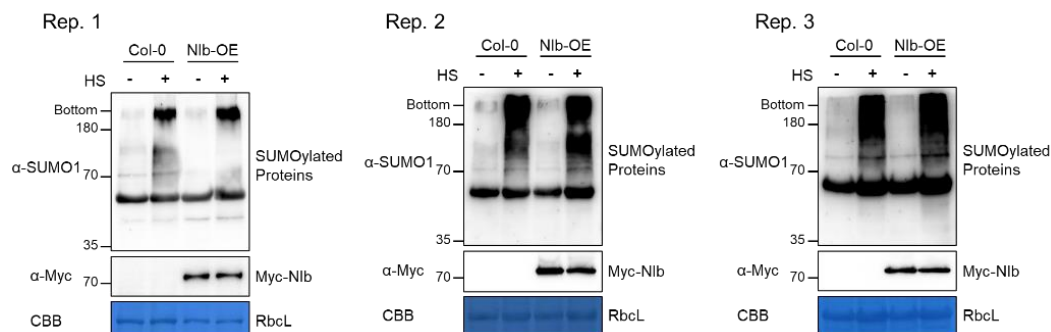
The paper by Ge et al. describes a novel mechanism to suppress host-mediated degradation of viral RNAs by the potyviral protein Nib. Overall the manuscript is well presented and the data shown substantiates the claims.

Response: Thank you for your very positive comments on our work and also for suggestions to strengthen this manuscript.

Major comments

The authors demonstrate using Y2H (Fig 2 and S2) that SCE1 interacts with different viral proteins (C1, NIa-VpG, NIa-Pro and Nib), but that only Nib appears to strongly suppress Pelota SUMOylation. Q: Did the authors investigate whether Nib inhibits the global SUMOylation response triggered e.g. by a heat shock in Arabidopsis? This test would demonstrate whether the inhibition of SCE1 activity by Nib is specific for Pelota only or generic inhibition. I missed this control.

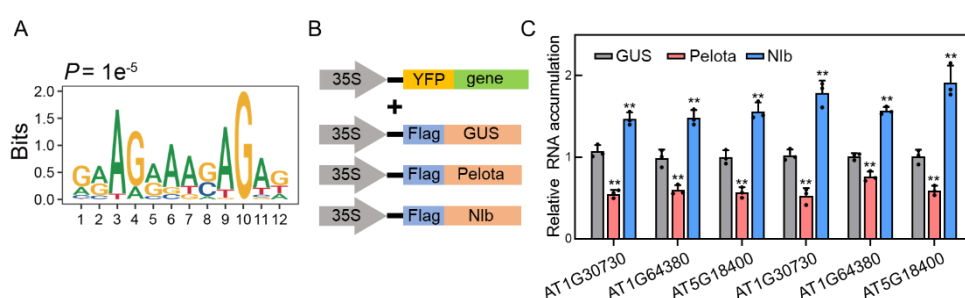
Response: We appreciate your suggestion. To address this concern, we examined the responses of SUMO levels to heat shock using anti-SUMO1 antibody in wild-type and Nib-transgenic plants. The global SUMOylation levels markedly increased under the heat shock condition (37 °C for 30 min) in Col-0 plants, but there were no obvious differences in Col-0 and Myc-Nib transgenic plants to heat shock responses. The experiment was repeated independently for three times, and we obtained a similar result. Therefore, we believe that Nib does not affect the global SUMO cascade activity, but specifically interferes with the interaction of SCE1-Pelota to affect the SUMOylation levels of Pelota. These data have been added in the revised manuscript (Supplementary Fig. 2b).



The authors argue that about 856 genes are upregulated both in the pelota null mutant and OE:Nib. Did the authors look if the corresponding mRNAs of the genes carry any (un)known signature/feature that would make these transcripts a target of Pelota degradation? Is the effect seen for the majority of these DEGs therefore indirect? In other words, is the effect on gene expression linked to increased mRNA transcription or due to reduced mRNA turnover?

Response: Thank you for these comments. To obtain a more detailed view of gene regulation, we searched for specific motifs in these enriched genes using MEME (<http://meme-suite.org/db/motifs>). The A-rich motif with high scores were considered the potential Pelota recognition site. Of note, this A-rich motif is similar with our previously identified Pelota recognition motif on TuMV RNA, which is GAAAAA, suggesting that the A-rich motif could be the Pelota recognition site of endogenous mRNA.

We hypothesized that the impact on gene expression was primarily due to mRNA turnover, similar to what occurs with TuMV RNA, since no enriched motifs were identified within the promoters of these genes. We selected 6 genes to test whether Pelota/Nlb could affect their RNA stability. In this study, we employed the CaMV 35S promoter to drive YFP-gene expression (35S: YFP was not regulated by Pelota/Nlb). YFP fusion genes co-expression with Flag-GUS, Flag-Pelota or Flag-Nlb together with P19 (a potent RNA silencing suppressor to exclude the effect of RNA silencing) in *N. benthamiana* leaves. As shown in the figure below, transient overexpression of Pelota decreased RNA accumulations, while Nlb increased them. Therefore, the A-rich motif is at least one of the Pelota recognition motifs in both the virus and plant transcripts. This A-rich motif was added in the revised manuscript (Supplementary Fig. 6c).



I missed in the Fig panels and/or methods how many times experiments were repeated independently. In particular, this holds for the IPs and in vitro protein expression/competition experiments. I strongly encourage the authors to upload all scans, images of the entire blots, films etc. for the various protein panels using FigShare or an alternative platform for the reader.

Response: Thank you for these valuable suggestions. The independently repeated times of experiments have been added as suggested in the revised manuscript. We have also provided all uncropped scans, images of the entire blots, films and other necessary information to meet the criteria of the journal (See the supplementary files).

Likewise for Fig 1A and 1D. The authors show two samples in the bar? Is this the number of repeats in a single experiment? Or is this average of one experiment with multiple biological samples? This applies for the other RT-qPCR samples as well in other figures. Please clarify the total number of samples analyzed (including technical versus biological repeats in a single experiment). And how many times the experiment was repeated independently.

Response: Thank you for pointing this out. Likewise for Fig 1a and 1d, we showed two groups in the bar that is the average of one experiment with multiple biological samples. To make this clearer, we have thoroughly checked the manuscript to ensure all error bars and statistics were appropriately defined, and we have added the necessary information on multiple biological samples and statistical tests used to all figure legends in the revised manuscript.

L142: Significant; Fig 1C does not show any quantification or statistical test meaning that the word 'significant' is not warranted. Please change the usage of this word across the entire manuscript. In fact, it is relevant to quantify, for example, the signal intensity ratios of the SUMOylated Pelota / Pelota-YFP in the IP. As the exposure times of the blots are different, it could be good to indicate this ratio (using the digital images of the ECL blots).

Response: Thank you for this comment. We provided all quantitative data (New Fig. 1c, 1e, 2a, 2b, 4c, 4d, 5c, 5e) in the revised manuscript based on this suggestion.

The experimental descriptions in the Materials and Methods is too brief in my opinion. (i) It is unclear to me e.g. in Fig 1F the authors mention a TuMV viral infected in combination with Pelota or Pelota/Nib 8 dpi. How is the Pelota and Nib expression performed? Using Agrobacterium? How many hours of expression etc. These details are missing in the caption and methods (or is known disconnected). (ii) I missed information on the plasmid design, backbones for the Agrobacterium transient expression system, the yeast two-hybrid system, plant transformation plasmids, in vitro expression etc. The reference in L532 is missing for the protoplast transfection. L585-586: how should I interpret the equal amount versus equal amounts. Please clarify the amounts used, protein concentrations, volumes etc. What is the composition of PBS in L594 etc. PBS can have different compositions, etc. The methods needs a strong revision to present the details needed to repeat the different experiments independently in another lab.

Response: We apologize for neglecting these details. (i) TuMV-GFP infectious clone was co-expressed with a mock control (Flag-GUS), Flag-Pelota, or Flag-Pelota and Flag-Nib in *Nicotiana benthamiana* by Agrobacterium infiltration. The inoculated plants were maintained to monitor symptom development and analysis of viral RNA accumulation in upper newly emerged leaves. Subsequent qRT-PCR analysis revealed that Pelota-mediated antiviral activity was significantly diminished in Nib transient-expression plants (Fig. 2f, g). (ii) These details have been provided in the revised manuscript. We have also completely revised the manuscript with more details and described the Materials and Methods and associated texts, to make readers understand and repeat these results easily.

The statistical test in several figures appears to be a student-t test while the authors compare multiple samples. Why did the authors decide for a t-test rather than an ANOVA combined with an appropriate post-hoc test (Fig 2c, 2e, 2g etc). L167 states that the ns means no significant difference, but it is unclear which statistical test was used. Please add all of this information throughout the entire manuscript.

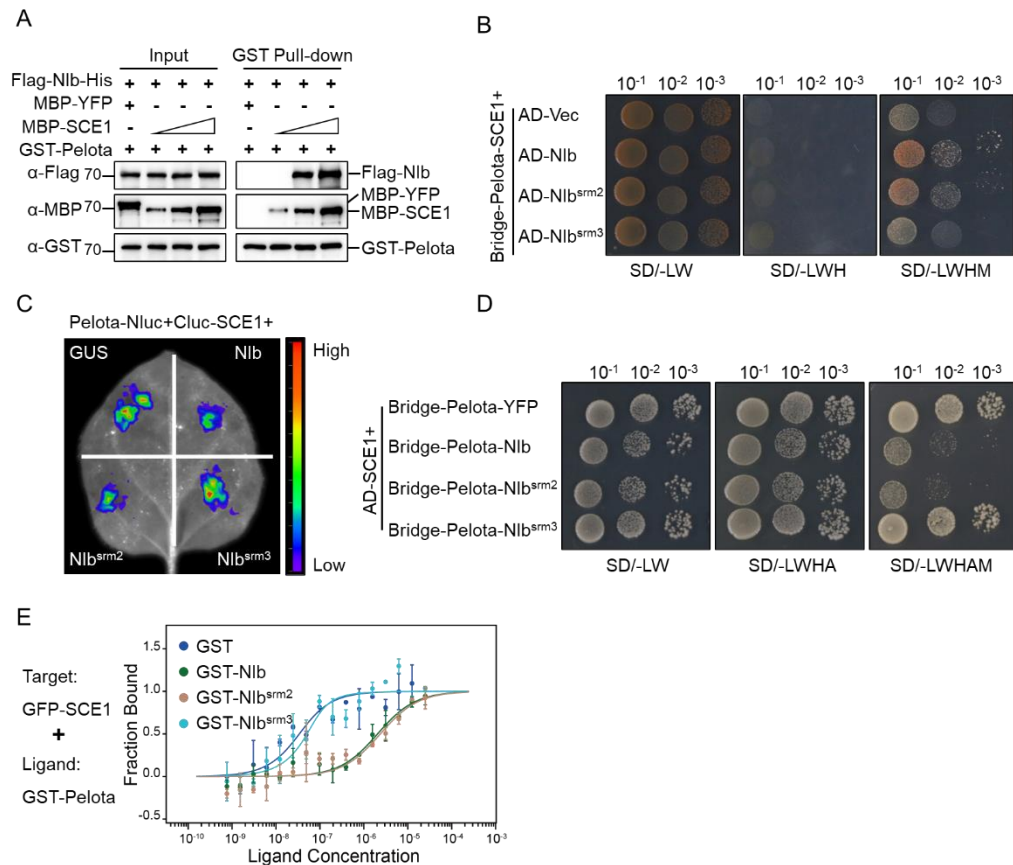
Response: We apologize for these unclear descriptions. As suggested, we have checked the manuscript to add all of this information. We have also provided the detailed description on how the data was processed in the revised manuscript. Multiple comparisons were performed using GraphPad Prism with one-way ANOVA followed by the recommended comparisons test. Pairwise comparisons were performed by two-tailed *Student's t* test.

L234-237: The authors claim Nib and Pelota do directly interact but that NI and Pelota form a complex (Fig 3c and 3e). The formation of a complex linked by SCE1 is not critical to suppress the SUMOylation of Pelota when SCE1 would be sequestered by Nib. To substantiate this claim of a protein complex, the authors could use the Y3H system to test if SCE1 acts as bridge between the 2 proteins. And is this bridge function is impeded when the NI SIM3 motif is mutated. As an alternative test to Fig 5e.

Response: Thank you for this valuable suggestion. We provided a Y3H analysis that demonstrated the interaction between Pelota and Nib using SCE1 as a bridge. Additionally, GST-Pelota was unable to pull down Nib without the presence of SCE1 (The following Fig. A-B). These results support the existence of Nib-SCE1-Pelota complex *in vivo*.

To better support this model, we provided additional experiments. We expressed Nib, Nib^{srn2}, and Nib^{srn3} in the Pelota-SCE1 LCI experiment (we changed SIM to SRM, SUMOylation-related motif, to avoid misunderstanding). It was observed that Nib and Nib^{srn2}, but not Nib^{srn3}, could obviously disrupt the fluorescence intensity of the interaction signal generated from Pelota and SCE1 (The following Fig. C). Y3H using Nib, Nib^{srn2}, and Nib^{srn3} as the bridge also showed a similar result (Following Fig. D). In addition, we also provided additional *in vivo* evidence using Microscale Thermophoresis (MST) to analyze the Pelota-SCE1 interaction. The interaction strength between Pelota and SCE1 was decreased in the presence of Nib and Nib^{srn2}, but not Nib^{srn3} (Following Fig. E).

These results support our model, that viral infection results in increased Nib accumulation, leading to Nib competing with Pelota for binding to SCE1. These data have been added in the revised manuscript (Supplementary Fig. 8d-f).



The authors demonstrate that mutating the sim2 in Nib already results in loss of virulence. Do the authors have an explanation? I missed a proper discussion on this finding.

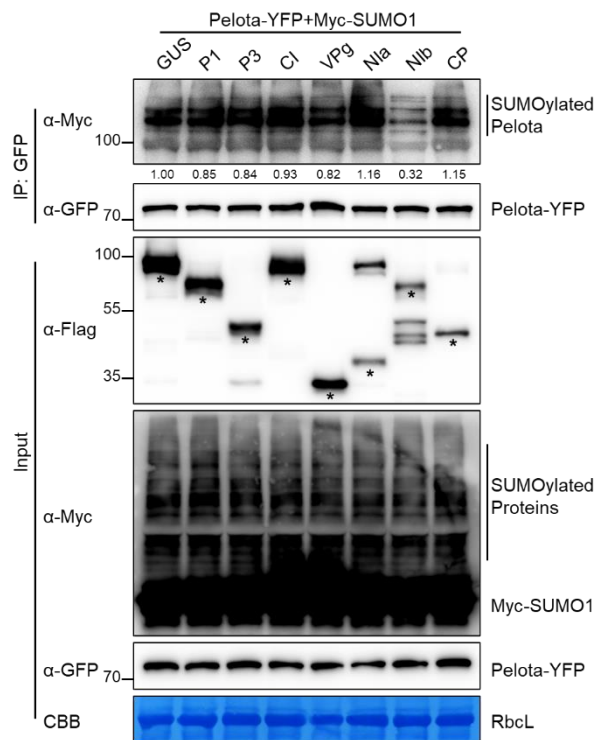
Response: Thank you for this comment. The SIM2 is crucial for Nib SUMO modification and viral virulence, which has also been reported previously (Cheng et al., 2017). In this study, we identified the Nib SRM3 is responsible for interacting with SCE1. Thus, Nib srm3 mutation not only lost the ability to interrupt Pelota-SCE1 interaction, but also interfere its self SUMOylation. We have added additional description about this citation to highlight this previous work.

Cheng X, Xiong R, Li Y, Li F, Zhou X, Wang A. Sumoylation of Turnip mosaic virus RNA Polymerase Promotes Viral Infection by Counteracting the Host NPR1-Mediated Immune Response. *Plant Cell*. 2017 Mar;29(3):508-525

Minor comments:

Fig2A: I missed a control for the protein levels of the different viral proteins; Are they expressed to the same level for all viral proteins?

Response: Thank you for pointing this out. We reconfirmed this result and provided evidence demonstrating the expression of all viral proteins.



L228 vigorous?

Response: We have corrected this in the revised manuscript.

L250 >Fig. 3b > Fig 3e

Response: We have corrected this in the revised manuscript.

L189: What is the expression assay used? pUC transfections?

Response: We used pJIT16318 for protoplast transformation, which contains a CaMV 35S promoter and a C-terminal GFP.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the authors for addressing our concerns.

1- Please explain the quantification of WBs. Was the respective amount of Pelota-YFP considered?

2- Please include replicates as supplemental figures. The information about biological replicates and quantification (eg Fig1) was also asked by another reviewer and it could be as well by readers.

3- The authors have addressed our comment that criticized the straightforward link made between mutating NIb and viral titer: “How can they be sure that the decrease in viral RNA titer is linked to inhibition of Pelota-mediated RQC and not a to a deleterious effect on viral replication?”. Their conclusion lines 390-1 “These results suggest that the mutated virus lost its capability to inhibit the Pelota-mediated RQC, which might result in ineffective infection to plants” is a bit more cautious and they have mentioned in the discussion the possibility that viral replication might be affected by the NIbsrm3 mutations. We still think it is misleading that this possibility is not mentioned in the results section. The authors observed a similar effect NIbsrm2 and NIbsrm3 mutation on virus titer, and this phenotype cannot be related to Pelota-mediated RQC.

Reviewer #2 (Remarks to the Author):

This extensive work shows that RNA quality control (RQC) is involved in limiting potyviral infection via the molecules involved in the NGD pathway. This antiviral activity requires the sumoylation of the Pelota protein, catalysed by SCE1 sumo-conjugating enzyme. The potyvirus RNA polymerase NIb acts as a decoy molecule for SCE1, reducing Pelota sumoylation and thus its antiviral activity. In the revised version of the manuscript NCOMMS-24-22626A, the authors have performed a substantial number of experiments in response to comments and criticisms from reviewers. They have successfully and adequately responded to my comments, and in general the results of the new experiments do not contradict the conclusions of the first version of the manuscript. I don't have any further comments.

Reviewer #4 (Remarks to the Author):

The authors have carefully answered all my suggestions. I have no more suggestions or comments. I appreciate the extensive effort made by the authors.

Point-by-Point Responses to Reviewers' Comments

Thanks to Dr. Wang for considering this manuscript. We also wish to thank four anonymous reviewers for providing us with constructive, insightful comments to improve the manuscript. A point-by-point response is provided for you to review. We hope that this revised version is acceptable for publication.

Editorial requests:

Reviewer #3 could not provide a review report and your responses were evaluated by the editorial team. First, we understand that the term 'SIM' is changed to 'SRM' to avoid potential confusion. However, given that these motifs are predicted to be SUMOylation interacting motifs based on residue sequence and have been referred to as 'SIM' in your earlier study about Nlb protein function (Cheng et al., 2017, Plant Cell), we suggest that you keep the terminology consistent and still use the term 'SIM'. On the other hand, we do agree with Reviewer #3 that it might cause some confusion that the SIMs, which are supposed to interact with SUMO, are investigated here for their potential interaction with SCE1. We think some explanation about the rationale behind such experimental design would be required.

Response: Thanks for these comments. We agree to maintain the use of the term 'SIM'. To avoid confusion, we have provided additional explanation about this experimental design and result within the manuscript.

Regarding the second major comment of Reviewer #3, new data dissecting the interaction among Nlb, SCE1, and Pelota are provided. We appreciate these new data but find it difficult to reconcile the Y3H data demonstrating the presence of a Nlb-SCE1-Pelota complex with the major claim that Nlb disrupts the interaction between SCE1 and Pelota as illustrated in the model in the last figure. We would ask that this apparent discrepancy is explained in the text.

Response: Thanks for this comment. We have added additional description about this. We think there is no obvious conflict between the existence of Nlb-SCE1-Pelota complex and the disruption of SCE1-Pelota by Nlb. When Nlb binds SCE1 from Pelota, there would have a co-existing state of the Nlb-SCE1-Pelota complex, that is why we could detect SCE1 as a bridge to connect Nlb-Pelota in the Y3H system.

Reviewer #1 (Remarks to the Author):

I thank the authors for addressing our concerns.

1- Please explain the quantification of WBs. Was the respective amount of Pelota-YFP considered?

Response: Thank you for this comment. The quantitative analysis of the specific WB band is based on the relative intensity to the control band. This has been detailed in the Method section.

2- Please include replicates as supplemental figures. The information about biological replicates and quantification (eg Fig1) was also asked by another reviewer and it could be as well by readers.

Response: Thank you for pointing this out. Done as suggested.

3- The authors have addressed our comment that criticized the straightforward link made between mutating NIb and viral titer: “How can they be sure that the decrease in viral RNA titer is linked to inhibition of Pelota-mediated RQC and not a to a deleterious effect on viral replication?”. Their conclusion lines 390-1 “These results suggest that the mutated virus lost its capability to inhibit the Pelota-mediated RQC, which might result in ineffective infection to plants” is a bit more cautious and they have mentioned in the discussion the possibility that viral replication might be affected by the NIbsrm3 mutations. We still think it is misleading that this possibility is not mentioned in the results section. The authors observed a similar effect NIbsrm2 and NIbsrm3 mutation on virus titer, and this phenotype cannot be related to Pelota-mediated RQC.

Response: We agree with this comment. By expressing NIb and its sim mutants, we could observe different changes in their effects on Pelota-mediated RQC (Fig. 5e-f and Fig. 6 d-e). However, as you mentioned, it is unclear whether the replicase function of NIb is impacted during viral systemic infection. We have described this possibility and discrepancy in the result section.

Reviewer #2 (Remarks to the Author):

This extensive work shows that RNA quality control (RQC) is involved in limiting potyviral infection via the molecules involved in the NGD pathway. This antiviral activity requires the sumoylation of the Pelota protein, catalysed by SCE1 sumo-conjugating enzyme. The potyvirus RNA polymerase NIb acts as a decoy molecule for SCE1, reducing Pelota sumoylation and thus its antiviral activity. In the revised version of the manuscript NCOMMS-24-22626A, the authors have performed a substantial number of experiments in response to comments and criticisms from reviewers. They have successfully and adequately responded to my comments, and in general the results of the new experiments do not contradict the conclusions of the first version of the manuscript. I don't have any further comments.

Response: We thank the reviewer for the positive assessment of our manuscript.

Reviewer #4 (Remarks to the Author):

The authors have carefully answered all my suggestions. I have no more suggestions or comments. I appreciate the extensive effort made by the authors.

Response: We thank the reviewer for the positive assessment of our manuscript.