EMBO reports

NS2 induces an influenza A RNA polymerase hexamer and acts as a transcription to replication switch

Junqing Sun, Lu Kuai, Lei Zhang, Yufeng Xie, Yanfang Zhang, Yan Li, Qi Peng, Yuekun Shao, Qiuxian Yang, Wen-xia Tian, Junhao Zhu, Jianxun Qi, Yi Shi, Tao Deng, and George Gao

Corresponding author(s): George Gao (gaof@im.ac.cn) , Yi Shi (shiyi@im.ac.cn), Tao Deng (dengt@im.ac.cn)

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

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Looking through the files, I would like to invite you to provide a final revised manuscript with the understanding that the remaining concerns of referee #2 must be addressed in the further revised manuscript and a final point-by-point response, as indicated in your appeal letter (by adding the new data mentioned in Addendum 1 and by discussing the related publications appropriately, in particular Zhang et al. J. Virol 2024 - PMID 38054704).

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- a short, two-sentence summary of the manuscript
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- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,

Achim

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Senior Editor
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Referee #1:

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In particular, a PDB validation report has now been submitted, which indicates a careful and technically flawless structure determination. The manuscript should hence be put forward for publication.

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All interpretations of NS2 functions must be based on solid experimental evidence. The simple mini-genome assays do not distinguish different mechanisms of function by NS2.

Point-by-point responses

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Looking through the files, I would like to invite you to provide a final revised manuscript with the understanding that the remaining concerns of referee #2 must be addressed in the further revised manuscript and a final point-by-point response, as indicated in your appeal letter (by adding the new data mentioned in Addendum 1 and by discussing the related publications appropriately, in particular Zhang et al. J. Virol 2024 - PMID 38054704).
Responses: We have addressed the remaining concerns of referee #2 in this point-by-point response letter. We have added Addendum 1 (in the appeal letter) in the manuscript as Appendix Figure S3 and discussed our recent related publications appropriately in Discussion (lines 345-348).

Moreover, I have these editorial requests.

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Responses: Completed.

- Please upload individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.
Responses: We have now provided as required.

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Responses: They have been revised as required.

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Responses: Completed.

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Responses: Completed.

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Responses: We have checked and modified as suggested.

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Responses: Completed

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Responses: They are all right.

- Thanks for providing source data (SD). You have been contacted already by our source data coordinator, who indicated which figure panels we would need source data for. However, the SD you have provided does not match to the checklist. I attach again the source data checklist. Please check and make sure that all the requested source data is provided. Moreover, please provide all the numerical data for panels 2D, 2E, 3E, 4E and 5C. Please upload all source data for one figure as one folder ZIPed together. Finally, please upload the filled in source data checklist with your final revised files.
Responses: Completed.

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- a short, two-sentence summary of the manuscript
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Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

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Response: We thank the reviewer for the encouraging comments on our manuscript.

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The authors interpret the function of NS2 as regulating viral RNA synthesis, ignoring its essential function as the nuclear-export protein.

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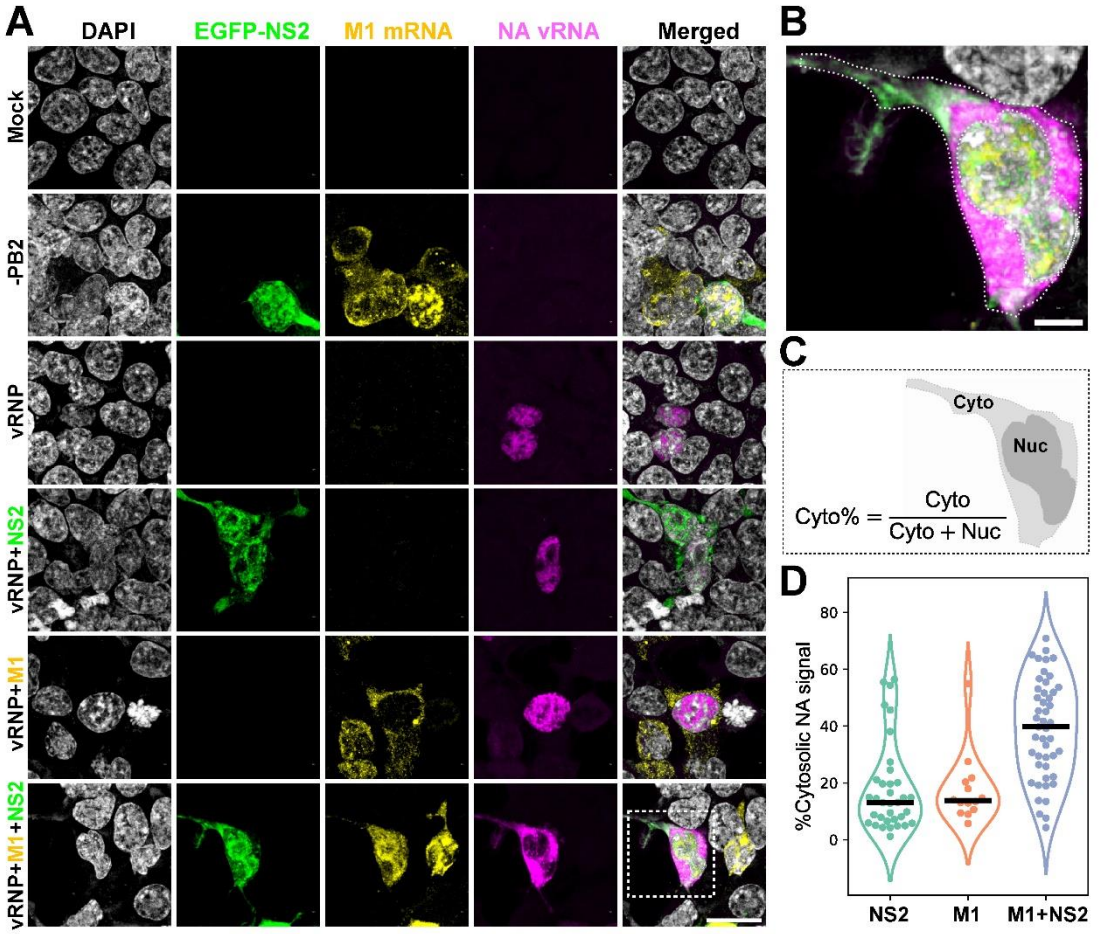
All interpretations of NS2 functions must be based on solid experimental evidence. The simple mini-genome assays do not distinguish different mechanisms of function by NS2.
Response: We are deeply sorry for our negligence in making this point clear in the original manuscript. According to the literature, the nuclear export of vRNP of influenza virus

requires forming a nuclear export complex of vRNP-M1-NS2 with host nuclear export protein Crm1 (*e.g.*, Martin & Helenius, 1991, O'Neill *et al.*, 1998, Paterson & Fodor, 2012). To confirm this generally accepted conclusion, we further conducted an *in-situ* FISH experiment under our experimental conditions (Addendum 1). It directly showed that, NS2 alone could not exert its function on vRNP nuclear export unless the M1 protein is co-expressed in the system. We have now included this result as Appendix Figure S3 in the manuscript as a validation of our system in studying the regulatory role of NS2 on transcription and replication (lines 208-216). Moreover, we have also added a paragraph in Discussion section to further exclude the concern and to discuss the different amino acids identified in the NES1 of NS2 NTD in exerting its independent functions on viral RNA syntheses and vRNP nuclear export (lines 359-372).



Addendum 1. The vRNPs are restricted in the nucleus when NS2 is expressed alone.

(A) HEK-293T cells were transfected with plasmids as indicated for 36 h and then probed against NA vRNA and M1 mRNA using Cy5 and Cy3 labeled single-molecule inexpensive FISH (smiFISH) probes. DAPI marks the cellular nuclei. Mock, no transfection. -PB2, the expression plasmids of M1, NS2 and vRNP except for PB2 protein were co-transfected. vRNP, the expression plasmids of vRNP were co-transfected. vRNP+NS2, the expression plasmids of vRNP and NS2 proteins were co-transfected. vRNP+M1, the expression plasmids of vRNP and M1 proteins were co-transfected. vRNP+M1+NS2, the expression plasmids of vRNP, M1, and NS2 proteins were co-transfected. Scale·bar, 20 μm. (B)

Zoomed in view of the vRNP+NS2+M1 co-transfection group, as marked by the dashed rectangle in (A). Scale·bar, 5 μm. (C) The efficiency of nuclear export of vRNP is determined by the ratio of vRNA in the cytosolic (Cyto%). (D) Quantification of the cytosolic fraction of vRNAs (Cyto%) of cells from the vRNP+NS2, the vRNP+M1, or the vRNP+M1+NS2 co-transfection groups.

References

Martin K, Helenius A (1991) Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell 67: 117-130

O'Neill RE, Talon J, Palese P (1998) The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. Embo J 17: 288-296

Paterson D, Fodor E (2012) Emerging roles for the influenza A virus nuclear export protein (NEP). Plos Pathogens 8: e1003019

Prof. George Gao
Institute of Microbiology,CAS
CAS Key Laboratory of Pathogen Microbiology and Immunology
No. 1 Beichen West Road,Chaoyang District
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China

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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Figures

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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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	**Yes**	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	