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Non-canonical proline-tyrosine interactions with multiple host proteins regulate Ebola virus infection

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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.)

1st Editorial Decision 25th Jun 2020

Dear Dr. Basler,

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, reviewers #1 and #3 appreciate the presented insights on EBOV VP30 interactions with multiple host proteins via an extended PPxPxY motif. However, all three reviewers indicate partially overlapping concerns regarding the experimental setup, data presentation and analysis, and highlight results that contradict with the currently proposed model (in particular points 5-6 by reviewer #2 and point c by reviewer #3). Furthermore, reviewer #3 indicates an important issue regarding the relevance of the individual and combinatory effect of the described VP30-host protein interactions for EBOV replication, also echoed in the point 6 by reviewer #1. Furthermore, reviewer #2 indicates that further experiments are needed to distinguish the effects on minigenome transcription vs replication (points 7-9 by reviewer #2).

Based on the interest expressed by reviewers #1 and #3, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and I would be happy to discuss the revision in more detail via email or phone/videoconferencing.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

With best regards,

Ieva

Ieva Gailite, PhD
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. Please note that the Data Availability Section is restricted to new primary data that are part of this study. If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc. in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 23rd Sep 2020.

Link Not Available

Referee #1:

This is an impressive body of work demonstrating that a proline rich motif in host cell proteins, which is also present in the Ebolavirus NP protein, binds to Ebolavirus VP30 thereby modulating viral transcription and replication. The study defines a novel extended proline rich binding motif PxPPPPxY in these proteins and shows by classical IP, elegant competition assays, mutagenesis approaches and hydrogen-deuterium exchanges studies that each of the proteins containing the motif binds to the same interface of VP30. This might affect VP30 phosphorylation and therefore viral transcription.
The study is of particular importance for virologists as it explains complex mechanisms regulating viral transcription through these interactions. Furthermore, this proline recognition domain might also play a role in regulating other cellular processes, with broad implications for cell biology as well as other diseases. Therefore, the study is of general significance and high priority for the EMBO readership.
In addition, the methodology and technical quality of the work seems high, however I have some suggestions for improvements and certain irregularities should be dissolved.

In particular:
- The authors argues that PEG10 is only expressed in Huh7.5 cells however a robust interaction can also be demonstrated in 293T cells (Fig.1D), even though expression levels are substantially lower.
- I think this is of importance since with this argument the authors avoid inclusion of PEG10 in experiments presented in Figs. 2B, where they perform siRNA mediated knockdown of the various factors, but not PEG10.
- Fig.2A: something is confusing with the WBlot here. Why is there a strong band in the HA-PEG10 labeled line even though it is the hnRNPL transfected sample; why are the levels for hnRNPL so different and what exactly do the authors mean when they write that the expression of the various proteins has differential effects on viral protein expression ("..demonstrated different degrees of inhibition of viral protein expression..")? I can not recapitulate this statement based on the data presented here. I am missing a loading control (tubulin)
- Fig.2B: knock-down of hnRNPUL1 does not result in reduced MG activity, which is somehow counterintuitive
- Fig.2B: please also perform WBlot analyses for NP, and VP35
- Fig.2C: please perform WBlots similar to Fig.2A/B; Furthermore it would be highly interesting to see combinatory effects of RBBP6 or hnRNP L knockdown in combination with hnRNPUL1 (activatory vs inhibitory); maybe this resolves the data from 2B, where they did not see an activating effect of hnRNPUL1 knockdown
- In general, and this applies to all figures and stats, the authors should not do statistics with triplicates and show just one representative experiments out of three biological replicates. All column diagrams should show the mean and standard deviation of the biological replicates (with appropriate stats). To show one representative WBlot is fine.
- Fig.3B: could the hnRNPUL1 peptide #3, the one that reduces MG activity, exert a dominant negative effect on the general activating properties of hnRNPUL1? The authors might like to assess this.
- Fig.6C: how can they calculate stats (and obtain significant results as indicated by the stars) with just two data points?

Referee #2:

The manuscript „Non-canonical proline-tyrosine interactions with multiple host proteins regulate Ebola virus infection" describes and characterizes a number of protein-protein interactions (PPI) of VP30 with the host proteins hnRNP L, hnRNPUL1, and PEG10. This work is based on a previous study which identified these PPI based on affinity purification and MS analysis. The authors confirm these PPI using CoIP assays, further refine information regarding the motifs bound by VP30, and demonstrate that the host proteins involved in these PPI modulate virus infection and reporter activity in minigenome assays, albeit in different ways, with hnRNP L and PEG10 impairing them, and hnRNPUL1 enhancing them.

Unfortunately, the study does not provide any further insight into the mechanism of how VP30-binding host factors modulate viral RNA synthesis, or why some of them appear to impair this process, while others enhance it. As such, the knowledge gain is incremental at best, particularly since the PPI themselves have already been previously identified and published, and in my view does not justify publication in EMBO. The model the authors suggest does not fit to all experimental data (see major points 5 and 6), and important experiments that would allow to test this model, and for which the authors have all necessary resources in place, have not been performed (see major points 8, 9 and 10). In addition, there are concerns particularly with respect to

the minigenome assays (see major points 1, 3, 4, 7), which are at the heart of the study assessing functional roles of the investigated host factors in the virus life cycle. In particular, the following points are problematic:

Major points:

1) For minigenome assays representative results from a single experiment out of at least three independent experiments are shown (e.g. in Fig. 2A, B, 3D), or information regarding the number of biological replicates / independent experiments are missing altogether (Fig. 2C). Since results from minigenome assays using luciferase as readout are easily and precisely quantifiable, this is peculiar, and raises questions with regard to experiment-to-experiment variation, as I would have expected the authors to integrate the data from the different experiments. Authors should either integrate the data from all experiments, or alternatively provide the raw data from all experiments as supplementary information. This is particularly important since the results from the minigenome assays are central to the overall conclusions of this study.

2) The labelling of Western Blots in Figure 2A is most likely incorrect; at least the band labelled HA-hnRNP is visible in all samples, not just the ones where this protein should be expressed. Further, the β-tubulin loading control is (at least judging by the labelling) missing here.

3) Results from Figure 2B and Figure 2C are inconsistent, although the standard deviation of values in these experiments is very low (e.g. single knockdown of RBBP6 results in 1200-fold increase in Figure 2B, but only 200-fold increase in Figure 2C). How do the authors explain this?

4) Also the way the minigenome data themselves are shown are peculiar, with data shown as fold increases compared to a -VP30 control. Standard in the field is to rather use -L controls, and then not to show fold increases (as -L controls should result in background noise), but rather absolute reporter values of the experimental samples and the -L control. Why do the authors deviate from this standard?

5) Figure 3B/D: For hnRNPL and hnRNPUL1 peptides minigenome results and CoIP results match, whereas for PEG10 they do not: PEG10 peptide 1 and peptide 2 show similar effects on minigenome activity, but only peptide 2 interacts with VP30, whereas peptide 1 does not. This casts doubt on the model the authors suggest, which postulates that interaction of the cellular factors with VP30 is responsible for modulation of RNA synthesis.

6) Similar doubts are cast by the finding that the P2A mutant in Figure 6D, which no longer is able to bind VP30, still inhibits reporter activity in a minigenome assay. While the authors disclose this, they do not provide a convincing explanation other than "a potential off-target effect".

7) Figure 7C: The authors try to assess transcription in a replication-competent minigenome. However, since in such an assay the number of vRNA templates available for transcription is dependent on replication, this assay does not allow any reliable conclusions regarding transcription alone to be made. The authors should rather use a replication-deficient minigenome which should be available to them as it was first developed in the Becker lab.

8) Doing so would indeed be important, since at least for hnRPUL1 it appears there is a strong effect on minigenome replication. Given that minigenome replication is an important contributor to overall reporter activity in a minigenome assay, this might explain why the authors see an increase in reporter activity after overexpression of hnRNPUL1. Therefore, it would be essential to assess

transcription and replication independently of each other!

9) Further, if the model the authors propose is correct, i.e. that interaction of these host factors modulates viral RNA synthesis, one would predict that, in the absence of VP30, knockdown or overexpression of these factors does not influence (mini-)genome replication. Demonstrating this would strengthen the proposed model, and is something the authors could easily do as they have all the tools available and the necessary assays established. All they would have to do is repeat the experiment shown in the bottom of panel 3D in absence of VP30, and they should observe no influence of overexpression of their host factors on vRNA levels (it might then be worthwhile to repeat this experiment also for siRNA knockdown of host factors).

10) Fig. 7D: Why do the authors assess the phosphorylation state of VP30 only after RBBP6 knockdown? They have all the resources available to do so for the other host factors, and since they suggest that interaction of these host factors disrupts the NP-VP30 interaction and prevents dephosphorylation of VP30 by PP2A B56, they should actually demonstrate this!

11) Experimental details are missing, e.g. the amounts of plasmid used in the minigenome assays, antibody concentrations, reagent amounts in the CoIP experiments, washing conditions, primer sequences for the RT-qPCR, transfection reagents and conditions in the minigenome assay, etc. Also, information regarding the fluorescence polarization is missing completely. It is currently impossible to repeat experiments, nor is it possible to thoroughly review the experimental data with so much crucial information absent.

Minor points:

1) Figure 2C: Why did the authors vary the amount of VP30 in this experiment?

2) Labelling of figures is sometimes not ideal. For example, in Figure 2B it is not clear what the 50 ng / 100 ng refer to (my guess is the amount of siRNAs), and in Figure 3D the relevance of the two bars only becomes apparent upon careful reading of the figure legend.

3) In Extended View Figure 3 PEG10 peptides 1 and 2 are evaluated as „+" and „+/-" for their effect in the minigenome assay. However, looking at the corresponding data in Figure 3D, the values for these two samples are virtually identical, so that this differentiation appears unjustified.

4) How do the authors explain the higher amounts of hnRNP L precipitated by the VP30 mutants D202A and Q229A?

Additional non-essential suggestions for improving the study:

1) In Fig 6C an explanation why certain combinations of point mutations were chosen would be helpful - at present they seem rather random.

Referee #3:

Batra et al present a second chapter to a manuscript published in 2018 where they used their methodology- expressing viral proteins and asking what do these expressed proteins interact with in cells. Previously they identified an interaction between the EBOV VP30 protein and the human

RBBP6 protein via a PPxPxY motif in the latter. In this manuscript they identify three other proteins, hnRNP L, hnRNPUL1 and PEG10 that interact with VP30 via the same mechanism. The biochemical characterization of the interactions between VP30 and these proteins (and some further analysis of RBBP6)is thorough and reveals interesting details about the requirements for the interaction between VP30 and these host factors. If these interactions are indeed important EBOV replication the work could be highly significant. The major concern is precisely whether or not the defined interactions are important for EBOV replication.

Specific major concerns:

1. While in Figure 7 the authors show that kd of hnRNP L and hnRNPUL1 enhance and repress EBOV replication respectively (measured in a surrogate GFP assay), the manuscript does not definitively determine whether or not the specific interaction studied is important for viral replication. I realize that this is a tough question to resolve but the authors should provide further evidence and/or deal with issues that raise questions about the physiological relevance of their findings:
a. No evidence is shown in the manuscript that VP30 interacts with the three newly identified host factors (or RBBP6 for that matter) during viral infection.
b. In Figure 2 the effect of the kd of proteins on the MG assay appears very variable between panels and does not completely agree with the hypothesis since kd of RBBP6 and hnRNP L results in similar level of enhancement and the double kd is not additive.
c.Peptide PEG10_1 has almost no affinity for VP30 and yet inhibits the MG assay as well as PEG10_2 (Figure 3). Yes this could be a non-specific inhibition of the assay, but it makes the rest of the data less certain. This is further confounded when in Figure 6D, other than GFP, no single peptide is negative in inhibiting this same MG assay.
d. How do the authors explain the different effects of hnRNP L, hnRNPUL1?

2. (I am sure this is an oversight) Figure 1D does not have a negative control.

3.

Minor concerns:
1. KD of RBBP6 appears to reduce level of all other proteins assayed - is there a significant reduction in cell number?
2. In Figure 3A the PPxPxY peptide from EBOV NP should be added to this figure for comparison.
3. In Figure 7A - kd of hnRNP L shows enhancement of infection, which I believe is different from data from Bukreyev and collaborators published in 2018. The authors should explain the disparity.

Referee #1:

- The authors argues that PEG10 is only expressed in Huh7.5 cells however a robust interaction can also be demonstrated in 293T cells (Fig.1D), even though expression levels are substantially lower.

- I think this is of importance since with this argument the authors avoid inclusion of PEG10 in experiments presented in Figs. 2B, where they perform siRNA mediated knockdown of the various factors, but not PEG10.

RESPONSE: We now include PEG10 in all our analyses. In binding assays, PEG10 clearly binds VP30 via its second PPxPxY motif (**Figs 1-4 and EV1**) and, like RBBP6 and hnRNP L, negatively impacts viral transcription (**Figs 6A, 6B, 7B and EV5A,B**) in over-expression studies. However, PEG10 knockdown does not significantly affect our EBOV minigenome (MG) assays (**Fig. 6C**) or significantly modulate EBOV infection (**Fig. 7**). We conclude that PEG10 is capable of modulating EBOV transcription when present at sufficiently high levels. However, at least in the cells tested, levels of PEG10 do not appear to be sufficiently high to have a demonstrable effect in our assays.

- Fig.2A: something is confusing with the WBlot here. Why is there a strong band in the HA-PEG10 labeled line even though it is the hnRNPL transfected sample; why are the levels for hnRNPL so different and what exactly do the authors mean when they write that the expression of the various proteins has differential effects on viral protein expression ("..demonstrated different degrees of inhibition of viral protein expression...")? I cannot recapitulate this statement based on the data presented here. I am missing a loading control (tubulin)

RESPONSE: We apologize for the confusion. The labels were misplaced in the original version. We have reordered how we present the data. The minigenome assays that was formerly **Fig. 2A** is now **Fig 6A**. The corresponding Western blot is now **Fig S1A**.

We have also clarified the text. We now write: Western blotting of lysates from these experiments demonstrated inhibition of viral protein expression at the higher concentration of hnRNP L, hnRNPUL1 and PEG10 plasmids (Fig. S1A). However, inhibition of viral protein expression was absent at the lower concentrations, where modulation of MG activity was still visible (Fig 6A, S1A).

- Fig.2B: knock-down of hnRNPUL1 does not result in reduced MG activity, which is somehow counterintuitive.

RESPONSE: We agree that several observations regarding hnRNPUL1 did not follow the pattern of the other interactors tested. Specifically, hnRNPUL1 overexpression

increases MG assay activity while over-expression of RBBP6, hnRNP L and PEG10 decrease MG assay activity. Knockdown of RBBP6 or hnRNP L increases MG activity and EBOV infection, whereas knockdown of hnRNPUL1 has little effect in the MG assay but modestly inhibits EBOV infection. To clarify the counterintuitive behavior of hnRNPUL1, we performed MG assays with a mutant minigenome RNA that, due to mutation of a specific stem-loop, functions independently of VP30. In this assay, RBBP6, hnRNP L and PEG10 lost their inhibitory activity upon over-expression. In contrast, hnRNPUL1 retained its capacity to enhance activity (**Fig EV5B**). We therefore conclude that hnRNPUL1 exerts activities that are independent of its binding to VP30.

- Fig.2B: please also perform WBlot analyses for NP, and VP35

RESPONSE: As requested, we now include Western blots for VP30, VP35 and NP upon siRNA knockdown. The minigenome assays that were formerly **Fig 2B** have been repeated and are now **Fig. 6C**. The corresponding Western blots are now **Fig S1C**. These data demonstrate that the knockdowns did not significantly alter expression of NP or VP35.

- Fig.2C: please perform WBlots similar to Fig.2A/B; Furthermore it would be highly interesting to see combinatory effects of RBBP6 or hnRNP L knockdown in combination with hnRNPUL1 (activatory vs inhibitory); maybe this resolves the data from 2B, where they did not see an activating effect of hnRNPUL1 knockdown

RESPONSE: **Fig 2C** has been moved **to Fig. 6D**. The corresponding Western blots are now **Fig S1D**.

As suggested by the reviewer, we tested the effects of RBBP6 or hnRNP L in combination with hnRNPUL1 in a MG assay. The data is provided here (**Fig 1 for reviewers)**. Titration of hnRNPUL1 in the presence of RBBP6 or hnRNP L or PEG10 resulted in decreased inhibitory activities of these proteins. Given our finding that the effects of hnRNPUL1 over-expression are VP30 independent (**See Fig EV5B**), we have chosen not to include this data in the revised manuscript.

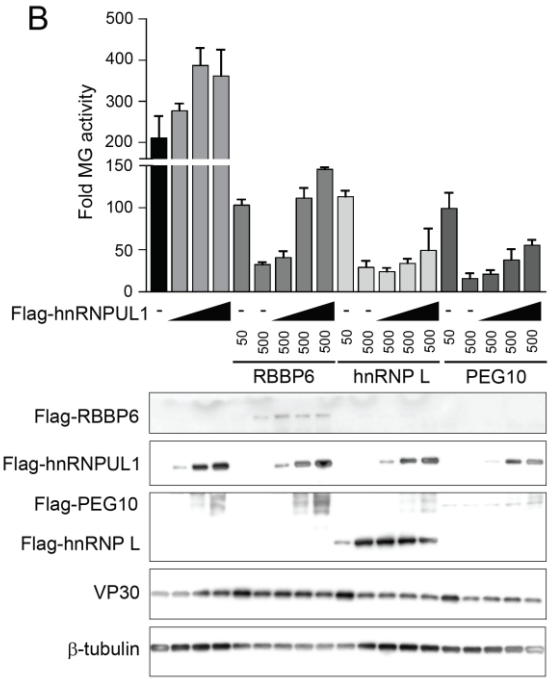

Fig 1 for reviewers. Mini-genome activity upon over-expression of RBBP6, hnRNP L or PEG10 in combination with hnNRPUL1. Data represent mean ± S.D. from one representative experiment (n=3) of at least two independent experiments.

We also tested the effect of hnRNPUL1 in combination with RBBP6 or hnRNP L upon siRNA knockdown. However, the double knockdowns were toxic to the cells. Therefore, this data is not included in the revised manuscript.

- In general, and this applies to all figures and stats, the authors should not do statistics with triplicates and show just one representative experiments out of three biological replicates. All column diagrams should show the mean and standard deviation of the biological replicates (with appropriate stats). To show one representative WBlot is fine.

RESPONSE: We apologize for the confusion and have clarified the figure legends. For each MG assay condition, three independent transfections were performed. The data presented are the mean ± the standard deviation (SD) for a set of triplicate transfections (n = 3). Each MG experiment presented was repeated at least 2 more times and yielded equivalent results.

- Fig.3B: could the hnRNPUL1 peptide #3, the one that reduces MG activity, exert a dominant negative effect on the general activating properties of hnRNPUL1? The authors might like to assess this.

RESPONSE: We thank the reviewer for the suggestion. We tested the effect of hnRNPUL1 peptide 3 on the general activating properties of hnRNPUL1. Peptide 3 indeed does exert a dominant negative effect on the activating properties of hnRNPUL1. We titrated GFP or GFP-peptide in the presence of hnRNPUL1 and GFP-peptide showed a strong inhibitory effect on MG activity. We have included this new data as **Fig EV5C**.

- Fig.6C: how can they calculate stats (and obtain significant results as indicated by the stars) with just two data points?

RESPONSE: We apologize for the confusion. We believe the reviewer was referring to the former **Fig 6D** which is now **Fig 5D**. As explained above, each MG experiment was performed with triplicate transfections for each condition (n=3). The experiment was repeated a second time and yielded the equivalent results.

Referee #2:

Major points:

1) For minigenome assays representative results from a single experiment out of at least three independent experiments are shown (e.g. in Fig. 2A, B, 3D), or information regarding the number of biological replicates / independent experiments are missing

altogether (Fig. 2C). Since results from minigenome assays using luciferase as readout are easily and precisely quantifiable, this is peculiar, and raises questions with regard to experiment-to-experiment variation, as I would have expected the authors to integrate the data from the different experiments. Authors should either integrate the data from all experiments, or alternatively provide the raw data from all experiments as supplementary information. This is particularly important since the results from the minigenome assays are central to the overall conclusions of this study.

RESPONSE: We apologize for the confusion and have clarified the figure legends. For each MG assay condition, three independent transfections were performed. The data presented are the mean ± the standard deviation (SD) for a set of triplicate transfections (n = 3). Each MG figure presented was repeated in the same fashion at least 2 more times and yielded equivalent results.

2) The labelling of Western Blots in Figure 2A is most likely incorrect; at least the band labelled HA-hnRNP is visible in all samples, not just the ones where this protein should be expressed. Further, the β-tubulin loading control is (at least judging by the labelling) missing here.

RESPONSE: We apologize for the confusion. The labels were misplaced in the original version. We have reordered how we present the data. The minigenome assays that was formerly **Fig. 2A** is now **Fig 6A**. The corresponding Western blot is now **Fig S1A**.

3) Results from Figure 2B and Figure 2C are inconsistent, although the standard deviation of values in these experiments is very low (e.g. single knockdown of RBBP6 results in 1200-fold increase in Figure 2B, but only 200-fold increase in Figure 2C). How do the authors explain this?

RESPONSE: The former **Fig 2B** and **2C** are now **Fig 6C** and **6D**. Transfection efficiencies vary from day to day. This accounts for the variation in fold-induction between **6C** and **6D**. As we have now clarified in the figure legends, for a given experiment, we perform each transfection three times in parallel and obtain numbers for this experiment. We then perform the same experiment at least two more times to ensure equivalent results are obtained.

4) Also the way the minigenome data themselves are shown are peculiar, with data shown as fold increases compared to a -VP30 control. Standard in the field is to rather use -L controls, and then not to show fold increases (as -L controls should result in background noise), but rather absolute reporter values of the experimental samples and the -L control. Why do the authors deviate from this standard?

RESPONSE: A lack of VP30 has also been shown to greatly reduce the signal in the mini-genome assay (Muhlberger et al. JVI 1999). Further, a number of previous published studies that, like ours, focused on VP30 function also used “no VP30” controls to calculate fold MG activity:

- Biedenkopf et al. J Virol. 2016 Apr 29;90(10):4914-4925. PMID: 26937028
- Martinez et al. J Virol. 2008 Dec;82(24):12569-73. PMID: 18829754
- Xu et al. Nat Commun. 2017 Jun 8;8:15576. doi: 10.1038/ncomms15576. PMID: 28593988
- Batra et al. Cell. 2018 Dec 13;175(7):1917-1930.e13. PMID: 30550789

Because our focus is on VP30, we viewed a “no VP30” condition as most the appropriate control. To address the concern raised by the reviewer, we performed a minigenome assay upon over-expression of host proteins and included both “no L” and “no VP30” controls to calculate fold activity (**Fig 2 for reviewers**). As depicted in the graphs, there is a slight difference in the background signal between “no VP30” and “no L”. However, the effect of host proteins on the MG activity is still substantial, follows the same pattern regardless of the control and does not alter the conclusions.

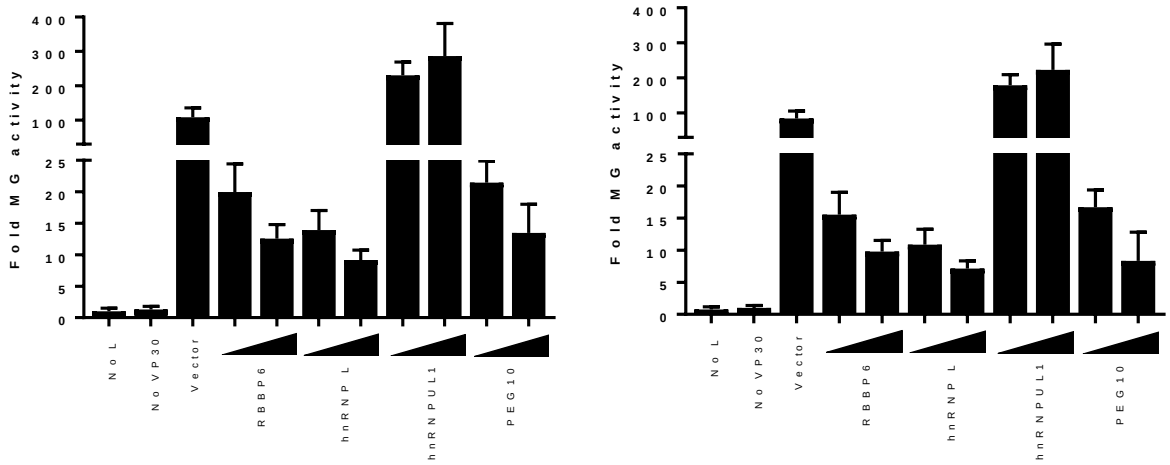

Fig 2 for reviewers. Minigenome assay upon over-expression of the indicated Flag-tagged host proteins. Fold activity was calculated relative to a no VP30 control (left) or a no L control (right).

5) Figure 3B/D: For hnRNPL and hnRNPUL1 peptides minigenome results and CoIP results match, whereas for PEG10 they do not: PEG10 peptide 1 and peptide 2 show similar effects on minigenome activity, but only peptide 2 interacts with VP30, whereas peptide 1 does not. This casts doubt on the model the authors suggest, which postulates that interaction of the cellular factors with VP30 is responsible for modulation of RNA synthesis.

6) Similar doubts are cast by the finding that the P2A mutant in Figure 6D, which no longer is able to bind VP30, still inhibits reporter activity in a minigenome assay. While the authors disclose this, they do not provide a convincing explanation other than "a potential off-target effect".

RESPONSE to points 5 and 6: We acknowledge that PEG10 peptide 1 (now in **Fig 6B**) and the RBBP6 P2A mutant data (now in **Fig 5D**) yield unexpected results. However, we disagree that the data obtained with these constructs casts doubt on other aspects of the study. Our proposed model is based on our demonstration of a common binding site on VP30 for each of the host factors, competition for binding with NP in vitro and in cell-based assays and the corresponding effects on VP30 phosphorylation. Given that these two peptide constructs are not produced naturally, we have not attempted to define the mechanisms by which they impact our assays.

7) Figure 7C: The authors try to assess transcription in a replication-competent minigenome. However, since in such an assay the number of vRNA templates available for transcription is dependent on replication, this assay does not allow any reliable conclusions regarding transcription alone to be made. The authors should rather use a replication-deficient minigenome which should be available to them as it was first developed in the Becker lab.

8) Doing so would indeed be important, since at least for hnRPUL1 it appears there is a strong effect on minigenome replication. Given that minigenome replication is an important contributor to overall reporter activity in a minigenome assay, this might explain why the authors see an increase in reporter activity after overexpression of hnRNPUL1. Therefore, it would be essential to assess transcription and replication independently of each other!

RESPONSE to points 7 and 8: We thank the reviewer for the valuable suggestions. As requested, we have now tested the effects of the host proteins on MG activity using the replication-deficient minigenome system developed by the Becker lab (**Fig EV5A**). Over-expression of RBBP6, hnRNP L and PEG10 resulted in a significant reduction in MG activity. However, hnRNPUL1 resulted in an increase in MG activity. These effects parallel the effects of each protein on the standard MG assay. These data suggest that each protein can modulate viral transcription independent of effects on replication.

9) Further, if the model the authors propose is correct, i.e. that interaction of these host factors modulates viral RNA synthesis, one would predict that, in the absence of VP30, knockdown or overexpression of these factors does not influence (mini-)genome replication. Demonstrating this would strengthen the proposed model, and is something the authors could easily do as they have all the tools available and the necessary assays established. All they would have to do is repeat the experiment shown in the

bottom of panel 3D in absence of VP30, and they should observe no influence of overexpression of their host factors on vRNA levels (it might then be worthwhile to repeat this experiment also for siRNA knockdown of host factors).

RESPONSE: In order to address the reviewer's concern, we tested the effects of the proteins on a MG system in which a stem loop at transcriptional start site has been mutated, allowing the MG assay to function in a VP30-independent manner (Weik et al. J Virol. 2002 Sep;76(17):8532-9. PMID: 12163572)(**Fig EV5B**). This mutant MG was not detectably affected by expression of RBBP6, hnRNP L or PEG10 in the absence of VP30. However, we observed a robust increase in minigenome activity upon over-expression of hnRNPUL1. This indicates that the effects of RBBP6, hnRNP L and PEG10 depend on the presence of VP30. However, the effects of hnRNPUL1 are independent of VP30 and do not require hnRNPUL1-VP30 interaction.

10) Fig. 7D: Why do the authors assess the phosphorylation state of VP30 only after RBBP6 knockdown? They have all the resources available to do so for the other host factors, and since they suggest that interaction of these host factors disrupts the NP-VP30 interaction and prevents dephosphorylation of VP30 by PP2A B56, they should actually demonstrate this!

RESPONSE: As suggested by the reviewer, we have assessed the phosphorylation levels of VP30 upon knockdown of RBBP6, hnRNP L and hnRNPUL1 in the context of MG assays (**now Fig 7C**) and in the context of EBOV infection (**now Fig 7D, E**). For both MG and infection experiments, we detected a significant decrease in pVP30 levels upon knockdown of hnRNP L or RBBP6. This is consistent with the robust effects of these knockdowns on MG activity and EBOV infection. In the MG assay, a very modest decrease was seen upon hnRNPUL1 knockdown. Given that hnRNPUL1 can exert effects on the MG assay in the absence of VP30, effects on the MG assay are likely unrelated to effects on VP30 phosphorylation.

11) Experimental details are missing, e.g. the amounts of plasmid used in the minigenome assays, antibody concentrations, reagent amounts in the CoIP experiments, washing conditions, primer sequences for the RT-qPCR, transfection reagents and conditions in the minigenome assay, etc. Also, information regarding the fluorescence polarization is missing completely. It is currently impossible to repeat experiments, nor is it possible to thoroughly review the experimental data with so much crucial information absent.

RESPONSE: We apologize for the missing information, we have now included all the experimental details including transfection and western blot conditions, primers for RT-qPCR etc.

Minor points:

1) Figure 2C: Why did the authors vary the amount of VP30 in this experiment?

RESPONSE: Our work is focused on to study the effect of host proteins on VP30 function, so we titrated different amounts of VP30 upon modulation of host protein expression to see any dose response.

2) Labelling of figures is sometimes not ideal. For example, in Figure 2B it is not clear what the 50 ng / 100 ng refer to (my guess is the amount of siRNAs), and in Figure 3D the relevance of the two bars only becomes apparent upon careful reading of the figure legend.

RESPONSE: We have revised and clarified the labelling in **Fig 6C** (formerly Fig 2B) and **Fig 6B** (formerly Fig 3D).

3) In Extended View Figure 3 PEG10 peptides 1 and 2 are evaluated as „+" and „+/-" for their effect in the minigenome assay. However, looking at the corresponding data in Figure 3D, the values for these two samples are virtually identical, so that this differentiation appears unjustified.

RESPONSE: We agree with the reviewer. Because we added substantial new data and needed to consolidate how the data was presented, we have removed this table from the manuscript. We feel that the re-organized presentation of the data makes the study easier to follow such that this summary table is not necessary.

4) How do the authors explain the higher amounts of hnRNP L precipitated by the VP30 mutants D202A and Q229A?

RESPONSE: As shown in our previous publications (Xu et al. Nat Commun. 2017 Jun 8;8:15576*;* Batra et al. Cell. 2018 Dec 13;175(7):1917-1930.e13), VP30 residues 202 and 229 are at the interface that interacts with PPxPxY motif. Mutations at these sites do not disrupt interaction and instead increase interaction with the PPxPxY host factors. It is not necessarily surprising that a change in a protein:protein interface might increase binding.

Additional non-essential suggestions for improving the study:

1) In Fig 6C an explanation why certain combinations of point mutations were chosen would be helpful - at present they seem rather random.

RESPONSE: Former **Fig 6C** is now **Fig 5C**. Because several mutants previously tested were not directly relevant to the conclusions drawn from this figure, these have been removed. We only compare binding to VP30 of the wildtype RBBP6 peptide and the P1,3,4 mutant because this mutant serves to validate the conclusions from single point mutant data presented in **Fig 5A and 5B**.

Referee #3:

1. While in Figure 7 the authors show that kd of hnRNP L and hnRNPUL1 enhance and repress EBOV replication respectively (measured in a surrogate GFP assay), the manuscript does not definitively determine whether or not the specific interaction studied is important for viral replication. I realize that this is a tough question to resolve but the authors should provide further evidence and/or deal with issues that raise questions about the physiological relevance of their findings:

a. No evidence is shown in the manuscript that VP30 interacts with the three newly identified host factors (or RBBP6 for that matter) during viral infection.

RESPONSE: Our data demonstrates a clear enhancement of EBOV infection upon hnRNP L knockdown (**Fig 7A**). This mirrors the effects of RBBP6 knockdown (Batra et al. Cell. 2018 Dec 13;175(7):1917-1930.e130). We now provide data demonstrating that knockdown of RBBP6 or hnRNP L decreases levels of VP30 phosphorylation in MG assays (**Fig 7C**) and in the context of EBOV infection (**Fig 7D and E**). These are the effects one would predict based on our in vitro and cell-based binding studies, where RBBP6 or hnRNP L compete with NP for binding to VP30. VP30-NP interaction promotes VP30 dephosphorylation to enhance viral transcription (Kruse et al. Mol Cell Mol Cell. 2018 Jan 4;69(1):136-145.e6). By removing a competitor, VP30-NP interaction would be increased, dephosphorylation would be more efficient and viral transcription would be enhanced. Together, these data demonstrate very clear effects of these proteins on EBOV infection and support our model.

Knockdown of hnRNPUL1 has the opposite effects as RBBP6 and hnRNP L upon EBOV infection. Given our new MG assay data where the effects of expressing hnRNPUL1 are VP30-indepenent (**Fig EV5B**), we propose that the effects of hnRNPUL1 on EBOV infection are also independent. Although PEG10 can bind VP30 and expression of PEG10 can inhibit MG activity, knockdowns have little impact in MG and EBOV infection assays. As we note in the discussion, we cannot exclude the possibility that PEG10 might have a demonstrable effect in cell types where PEG10 expression is higher.

b. In Figure 2 the effect of the kd of proteins on the MG assay appears very variable between panels and does not completely agree with the hypothesis since kd of RBBP6 and hnRNP L results in similar level of enhancement and the double kd is not additive.

RESPONSE: We have consistently observed substantial increases in MG activity upon RBBP6 or hnRNP L knockdown. We also consistently see a further increase in MG activity upon double knockdown. It is based on this observation that we conclude the effects of double knockdown are roughly additive.

c. Peptide PEG10_1 has almost no affinity for VP30 and yet inhibits the MG assay as well as PEG10_2 (Figure 3). Yes this could be a non-specific inhibition of the assay, but it makes the rest of the data less certain. This is further confounded when in Figure 6D, other than GFP, no single peptide is negative in inhibiting this same MG assay.

RESPONSE: We acknowledge that PEG10 peptide 1 (now in **Fig 6B**) yielded unexpected results. However, we disagree that the data obtained with these constructs casts doubt on other aspects of the study. Our proposed model is based on our demonstration of a common binding site on VP30 for each of the host factors, competition for binding with NP in vitro and in cell-based assays and the corresponding effects on VP30 phosphorylation. Given that these peptide constructs are not produced naturally, we have not attempted to define how they impact our assays.

d. How do the authors explain the different effects of hnRNP L, hnRNPUL1?

RESPONSE: hnRNPUL1 overexpression increases MG assay despite its capacity to bind VP30 and compete with NP for VP30 binding. To clarify the counterintuitive behavior of hnRNPUL1, we performed MG assays with a mutant minigenome RNA that, due to mutation of a specific stem-loop, functions independently of VP30. In this assay, RBBP6, hnRNP L and PEG10 lost their inhibitory activity upon over-expression in the absence of VP30. In contrast, hnRNPUL1 retained its capacity to enhance activity (**Fig EV5B**). We therefore conclude that hnRNPUL1 exerts activities that are independent of its binding to VP30.

2. (I am sure this is an oversight) Figure 1D does not have a negative control.

RESPONSE: We apologize for the confusion, but it does have a negative control: the IP of empty vector-transfected cells. The anti-HA antibody only pulls down the host protein when HA-VP30 is present and not when it is absent. We have clarified this in the text, writing: Endogenous RBBP6, hnRNP L and hnRNPUL1 were each co-immunoprecipitated in the presence of HA-VP30 but not in the presence of empty vector (Fig. 1D).

Minor concerns:

1. KD of RBBP6 appears to reduce level of all other proteins assayed - is there a significant reduction in cell number?

RESPONSE: RBBP6 is an essential gene with crucial functions during cell proliferation and differentiation (Ntwasa 2016, *Trends in Cancer*). However, we did not observe any significant reduction in cell number upon RBBP6 knockdown.

2. In Figure 3A the PPxPxY peptide from EBOV NP should be added to this figure for comparison.

RESPONSE: This is now **Fig 2A**. As requested, we have added the EBOV NP sequence.

3. In Figure 7A - kd of hnRNP L shows enhancement of infection, which I believe is different from data from Bukreyev and collaborators published in 2018. The authors should explain the disparity.

RESPONSE: We have shown through series of experiments that hnRNP L negatively regulates EBOV replication. In Bukreyev et al., knockdown of hnRNP L showed inhibition of virus infection (Fig.1C in their paper). However, the siRNAs used in the study seem to be very toxic to the cells and siRNA#2 which showed significant inhibition of virus infection also resulted in more than 50% reduction in cell numbers (as shown in their supplementary figure S3). Further the authors did not show the knockdown efficiency in their experiments.

1st Revision - Editorial Decision 26th May 2021

Dear Chris,

Thank you for submitting a revised version of your manuscript. Your revised study has now been seen by two of the original referees. While they find that most of their concerns have been addressed, they also indicate overlapping issues with data presentation and the statistical analysis. Therefore, I would like to invite you to address these concerns in the final revision round by adding the requested information on statistical analysis and either including all experiments in the analysis or adding the data from the independent replicates in the Appendix.

Additionally, please address the following editorial points:

1. Please either add more detailed information in the Author Checklist section C or indicate where this information can be found in the manuscript.
2. Figure panel 5D is not mentioned in the text, please add a callout.
3. Due to its large size, please split Figure EV3 into two figures - each figure has to fit a single A4 page.
4. Please remove figure legends from the figure files.
5. Please remove the Appendix Figure legends from the main manuscript text file and add them to the Appendix file together with a short table of contents.
6. Please adjust the reference format to the journal style (up to 10 authors before et al, https://www.embopress.org/page/journal/14602075/authorguide#referencesformat).
7. We require a "Data Availability" section at the end of Materials and Methods. As far as I can see, no data deposition in external databases is needed for this paper. If I am correct, then please state in this section: "This study includes no data deposited in external repositories".
8. We generally encourage publication of source data for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as supplementary "Source Data". Please let me know if you have any questions about this policy.
1. Based on the already provided source data, the Western blot bands for most of the panels in figures EV1 and EV2A, C, E have been significantly altered. Please adjust the panels to reflect the original dimensions of the bands.
9. Tubulin panels in Fig EV2C, E do not appear to fit to the provided source data - please check.
10. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the size is rather small and the text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Please let me know if you have any further questions regarding any of these points. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version.

With best regards,

Ieva

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Further information is available in our Guide For Authors:
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 24th Aug 2021.

Link Not Available

Referee #1:

The authors adequately responded to my (as well the other reviewers) comments and did several new experiments to strengthen their conclusions and claims. Furthermore they restructured the manuscript improving its readability. Altogether, the manuscript contains an impressive amount of data firmly establishing that the novel proline motif in VP30 interacts with host cell factors to modulate viral transcription.
Apart from this enthusiasm, I was really disappointed how they responded to my concern related to the statistical analyses. Reviewer 2 also brought this up. As far as I am trained in statistics, it is not allowed to do statistics with triplicates, ie three independent transfections/infections done in parallel. This is not defined as a biological replicate. Furthermore, for several caluclations they miss to give the number of replicates and/or to indicate the statistical test used. Sorry to say, this is embarrassing given that such experienced and renowned scientists are listed as authors and co-authors.
Fig 4D: n is missing; statistical test used not indicated
Fig 5D: n is missing; statistical test used not indicated
Fig 6A-C: stats calculated with triplicates, not biological replicates; statistical test used not indicated
Fig 7B: duplicates and biological replicate data were grouped to calculate stats; this is n=2 not n=4
Fig EV2E: stats calculated with triplicates, not biological replicates; statistical test used not indicated
Fig EV5A-C: stats calculated with triplicates, not biological replicates; statistical test used not indicated

Referee #2:

This is a revised version of a previously submitted manuscript. The authors have addressed most of my (reviewer #2) concerns. However, I still consider it very problematic that in case of the minigenome experiments they only provide data from three biological replicates from a single experiment (response to former major point 1). This is of particular concern in light of the information that the authors see dramatic experiment-to-experiment variations (response to major point 3). I realize that complex cell-based assays might sometimes exhibit such variations due to small experimental differences (e.g. transfection efficacy or cell density at the time of transfection) that are difficult to control. One way to deal with those differences would be to normalize results to a control (i.e. setting the positive control to 100%); however, this might be not compatible with the authors way of presenting the data as fold-differences compared to -VP30. At the very least the authors should report the results from all experiments, e.g. as supplementary information, even if they opt not to integrate all data in a single figure!

Further, as a minor comment while the authors in most figure legends now disclose that data are derived from a single experiment, in the legend to figure 5D this is not the case.

Response to Editor and Referee Comments

Editor's Comments:

Thank you for submitting a revised version of your manuscript. Your revised study has now been seen by two of the original referees. While they find that most of their concerns have been addressed, they also indicate overlapping issues with data presentation and the statistical analysis. Therefore, I would like to invite you to address these concerns in the final revision round by adding the requested information on statistical analysis and either including all experiments in the analysis or adding the data from the independent replicates in the Appendix.

We now provide additional replicates of each minigenome assay in the paper as Appendix Figures.

Additionally, please address the following editorial points:

1. Please either add more detailed information in the Author Checklist section C or indicate where this information can be found in the manuscript. This has been added.

2. Figure panel 5D is not mentioned in the text, please add a callout: We have indicated Figure 5D in the text.

3. Due to its large size, please split Figure EV3 into two figures - each figure has to fit a single A4 page.: As suggested, we have split Figure EV3 into two, EV3 and EV4.

4. Please remove figure legends from the figure files: We have removed the figure legends from figure files and have included them in the text file.

5. Please remove the Appendix Figure legends from the main manuscript text file and add them to the Appendix file together with a short table of contents. All the appendix figures along with a table of contents and appendix figure legends are included as one Appendix file.

6. Please adjust the reference format to the journal style (up to 10 authors before et al, https://www.embopress.org/page/journal/14602075/authorguide#referencesformat). The references have been formatted to the journal style.

7. We require a "Data Availability" section at the end of Materials and Methods. As far as I can see, no data deposition in external databases is needed for this paper. If I am correct, then please state in this section: "This study includes no data deposited in external repositories". This study has no data deposited in the external databases. We have indicated this in the methods section.

8. We generally encourage publication of source data for electrophoretic gels and blots, with the

aim of making primary data more accessible and transparent to the reader. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as supplementary "Source Data". Please let me know if you have any questions about this policy.

We have included source files for all of the western blots in the paper.

1. Based on the already provided source data, the Western blot bands for most of the panels in figures EV1 and EV2A, C, E have been significantly altered. Please adjust the panels to reflect the original dimensions of the bands. We have adjusted the panels and matched the original dimensions of the bands in the figures EV1 and EV2.

9. Tubulin panels in Fig EV2C, E do not appear to fit to the provided source data - please check. The tubulin panels were stretched and resized, we have now changed these panels to match the source files.

10. Papers published in The EMBO Journal are accompanied online by a 'Synopsis' to enhance discoverability of the manuscript. It consists of A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height, jpeg or png format). You can either show a model or key data in the synopsis image. Please note that the size is rather small and the text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We have included a synopsis figure along with a short summary and key results.

Referee #1:

The authors adequately responded to my (as well the other reviewers) comments and did several new experiments to strengthen their conclusions and claims. Furthermore, they restructured the manuscript improving its readability. Altogether, the manuscript contains an impressive amount of data firmly establishing that the novel proline motif in VP30 interacts with host cell factors to modulate viral transcription.

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Fig 4D: n is missing; statistical test used not indicated

Fig 5D: n is missing; statistical test used not indicated
Fig 6A-C: stats calculated with triplicates, not biological replicates; statistical test used not indicated:
Fig 7B: duplicates and biological replicate data were grouped to calculate stats; this is n=2 not n=4
Fig EV2E: stats calculated with triplicates, not biological replicates; statistical test used not indicated
Fig EV5A-C: stats calculated with triplicates, not biological replicates; statistical test used not indicated

We thank the reviewer for the positive comments. To address reviewer's concern about the statistical analysis, we have now included additional replicates for minigenome assays as appendix figures. For each MG assay experiment, the data presented are the mean ± the standard deviation (SD) for a set of triplicates (n = 3) done in parallel. Each MG figure presented was demonstrated to be reproducible by additional experiments. The graphs included in the Appendix demonstrate this.

We apologize for the confusion and have clarified the details about the statistical tests and replicates in the figure legends for all of the figures, as pointed out by the reviewer. Of note, we have split figure EV3 into EV3 and EV4, so former figure EV4 is EV5 and figure EV5 is now EV6.

Referee #2:

This is a revised version of a previously submitted manuscript. The authors have addressed most of my (reviewer #2) concerns. However, I still consider it very problematic that in case of the minigenome experiments they only provide data from three biological replicates from a single experiment (response to former major point 1). This is of particular concern in light of the information that the authors see dramatic experiment-to-experiment variations (response to major point 3). I realize that complex cell-based assays might sometimes exhibit such variations due to small experimental differences (e.g. transfection efficacy or cell density at the time of transfection) that are difficult to control. One way to deal with those differences would be to normalize results to a control (i.e. setting the positive control to 100%); however, this might be not compatible with the authors way of presenting the data as fold-differences compared to -VP30. At the very least the authors should report the results from all experiments, e.g. as supplementary information, even if they opt not to integrate all data in a single figure!

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We apologize for the missing the statistical details for Figure 5D. We have now added the details regarding the statistical analysis to the figure legend.

2nd Revision - Editorial Decision 9th Jul 2021

Dear Dr. Basler,

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

Congratulations on a very nice study.

Yours sincerely,

David del Alamo
Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
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Corresponding Author Name: Christopher F. Basler
Journal Submitted to: EMBOJ
Manuscript Number: EMBOJ-2020-105658

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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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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?	Measurements were performed in triplicate and statistical tests applied to evalute significance. ANOVA with Tukey's multiple comparions test was used unless otherwise indicated. This approach is based on past experience with the assays performed and the standard approach used in the literature. We provide additional repeats of the minigenome assays as Appendix figures to further demonstrate the validity of our conclusions.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable
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.	The data are assessed based on reproducibility and statistical significance. Subjective bias should not be relevant.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
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	Not applicable
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.	Data was considered significant if p<0.05.

Is there an estimate of variation within each group of data?	We assessed standard deviation among samples.
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).	Catalog numbers for all commercially-available antibodies used are provided in the Antibodies section of Materials and Methods. Citations are provided for the anti-VP30, anti-phospho VP30 and anti-NP antibodies in the Antibodies section of Materials and Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The sources of all cell lines used are provide in the Cell Lines section of Material and Methods. As indicated, the cell lines were tested regularly for mycoplasma contamination. The cell lines were not authenticated by STR profiling after receipt from the source.

* 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.	Not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable
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.	Not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
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.	Not applicable
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.	Not applicable

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	Not applicable
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).	None of our data fall into the mandatory category. If accepted we can make original data available.
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).	Not applicable
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.	Not applicable

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