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Host succinate inhibits influenza virus infection through succinylation and nuclear retention of the viral nucleoprotein

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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.)

Dear Dr Si-Tahar,

Thank you for the submission of your manuscript (EMBOJ-2021-108306) to The EMBO Journal. Your study has been initially sent to three reviewers, however one reviewer got much delayed and, in the end, did not send us his-her report even after repeated chasers. We have received feedback from the other two referees, which I enclose below, and decided to proceed with our decision based on these reports.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of major issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. In more detail, the experts state that for various parts of the data presented, orthogonal methodologies and complementary control experiments are critically required to consolidate the findings. A particular point of attention are the apparent differences in antiviral activity observed in vitro and in vivo (Ref#1, pts.2,3).

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

In light of the extensive experimentation requested by the reviewers, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for revisions.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When submitting your revised manuscript, please carefully review the instructions below.

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

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) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see

https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** 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.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

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Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 12th Jan 2022:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

Guillon et al.,

Scope

Guillon et al., describe the relationship between the TCA-metabolite succinate and infection with the pathogenic virus IAV in both humans and mice. The authors claim that infection with IAV in mice and in humans leads to the accumulation of succinate. Further, that succinate has anti-viral effects that involve succinylation of viral factor nucleoprotein (NP).
The topic is very timely and the data are quite novel. Only few other reports demonstrate a direct anti-viral effect of host metabolites with effect in vivo as well as in vitro. In general, the data support the claims made by the authors - although extra methods could in several cases be involved to substantiate the data. The manuscript represents a significant scientific advance within this novel field of research which could have clinical implications for development of new treatments for IAV.

Major
1) The setup involves addition of succinate 4hours post infection. I think it is a little unclear why. If the idea is to test if succinate works post entry - then why wait 20h before harvest. The replication cycle of IAV is much shorter than 20hours (6-8 as I recall). So, by waiting 20hours before harvest, it is difficult to estimate if the effect was after entry for first round of replication - or through an entry related event in the next generations. I cannot out-rule that I am missing point her - but this must be addressed.

2) Figure 2. It is a bit surprising that the effect of succinate in vitro is much less that observed in vivo (50% vs several logs). Normally, I would assume that delivery of succinate would be easier in vitro and thus better effect. It would be great to test succinate in vitro across airway epithelial cell-lines.

3) Figure 4. This figure would benefit from additional data on in vivo virus load. These should include qPCR data for virus mRNA and Western blotting for virus protein on lung samples.

4) Figure 5. Again, related to the timing. The is no observation of difference in mRNA. Even after 24 hours. But here we assume that less virus must be released from the succinate-treated cells. Thus, we must assume that fewer cells are infected from progeny virus. Again, I cannot out-rule that I am missing point her - but this must be addressed.

5) Figure 7. The conclusion that NS1 is not involved in the effect of succinate cannot be made without thorough comparison of the effect between the two virus strains. It looks like the reduction by succinate is lower than previously observed. The data here are too few to make that conclusion. Further, is there an effect of succinate on virus replication in the delta-NS1 virus strain?

6) Figure 8. Many controls to very the NP-vRNA antibody are missing. Uninfected controls for example. Futher, I suggest a simple pull-down experiment to test if the NP-vRNA antibody pulls down less vRNA and NP in succinate treated cells vs controls.

Referee #3:

The finding that succinate inhibits influenza virus infection through succinylation and nuclear retention of the viral nucleoprotein is interesting and important to understand the life cycle of influenza A viruses. It may also provide new ground to develop antivirals. The data provided support the conclusions, but as outlined below, a series of important control experiments are required.

Major points:

1.) Fig. 3c: From the data provided the inhibition of viral replication by succinate treatment is rather unclear. Two-fold changes are very low for a virus that can replicate up to 10 to 8 PFU/ml. The authors should provide more information by performing single and multitype growth curves. If the viral inhibition is only in the two-fold range it is difficult to believe the EM results shown in Fig. 3d.

2.) Fig.4: In Fig. 4d virus is mixed with succinate and then applied to the animal. For a clear interpretation of this in vivo experiment, it is important to know whether mixing the virus with succinate inactivates the virus. Partial and complete inactivation might have caused the extreme variation in lung titers 4 days post infection. Cell culture experiments (multiple growth curves) can be performed to find out whether succinate inactivates viral particles also directly. If this is the case, the in vivo experiments show in Fig. 4 are not conclusive.

3.) Fig. 4: The K87R mutant virus should show no effect in mice when combined with succinate. Prior in vivo infection experiments the authors should perform growth kinetics (multiple growth curves) whether wt and K87R mutant virus grow similar and whether K87R is affected by succinate treatment.

4.) Fig. 6: In panel a the authors show complete retention of NP signal in the cell nucleus after 20 h. This would clearly not be compatible to only 50-60% inhibition in viral growth. Since IF analysis are often biased, cellular fractionation and subsequent WB analysis should be performed using NP and other antibodies to demonstrate the extend of nuclear retention.

5.) Fig. 8: Cell fractionation and WB analysis are required to support the IF data set. Please also provide viral titers at the

respective timepoints.

6.) The authors speculate that treatment with succinate causes a packaging defect. This can be easily shown.

Minor points:

1.) Fig. 8a: In this panel it is indicated that the antibody recognizes NP and the viral RNA. Was this experimentally shown or do the authors want to say that the antibody recognizes specifically NP within a vRNP complex?

2.) The literature regarding other post translational modifications of IAV proteins and in particular NP is not discussed.

Dear Dr. Klimmeck,

We thank you and the reviewers for the thoughtful review of our manuscript entitled: ***"Host succinate inhibits influenza virus infection through succinylation and nuclear retention of the viral nucleoprotein"***
We also appreciate the positive comments of the referees regarding our article.

We hope that our clarifications will make it now suitable for publication in the *EMBO Journal.*
Please find below our point-by-point response to the reviewers' concerns as well as the changes we made in the revised manuscript (marked with a red color).

Referee #2:

General comment

The topic is very timely and the data are quite novel. Only few other reports demonstrate a direct anti-viral effect of host metabolites with effect in vivo as well as in vitro. In general, the data support the claims made by the authors (…) The manuscript represents a significant scientific advance within this novel field of research which could have clinical implications for development of new treatments for IAV.

We thank the reviewer #2 for her/his positive opinion and constructive input.

1) The setup involves addition of succinate 4hours post infection. I think it is a little unclear why. If the idea is to test if succinate works post entry - then why wait 20h before harvest. The replication cycle of IAV is much shorter than 20hours (6-8 as I recall). So, by waiting 20hours before harvest, it is difficult to estimate if the effect was after entry for first round of replication - or through an entry related event in the next generations. I cannot out-rule that I am missing point her - but this must be addressed.

We agree with the reviewer that the choice of this protocol setting has to be clarified. In our initial experiments, we assessed whether lung epithelial cells had to be exposed to succinate prior or concomitantly to IAV infection or even after IAV challenge. We further tested several incubation periods of succinate post-IAV infection to "mimic" a curative intervention. We found that 4h was a time point at which succinate could still induce an antiviral activity.
Besides, we also have to underline that in our experimental conditions, lung epithelial cells were challenged with an amount of virus (MOI=1) so that all cells were infected approximately

simultaneously and limited to a single cycle of infection. Thus, in absence of the adequate proteases in the extracellular medium, the released neovirions cannot reinfect non-infected surrounding cells.

Nevertheless, we also tested the effect of succinate on IAV-infected lung epithelial cells under experimental conditions (MOI=10^{-3}; presence of TPCK-treated trypsin) that support multicycle replication of the virus.

We confirmed and extended our previous findings by showing that succinate impairs IAV multiplication even more strongly in multicycle replication condition (~5-fold decrease) than in single replication cycle condition (~2-fold decrease), as evidenced by a plaque-forming unit (PFU) assay (see the new Figure EV1, panels (a) and (b)).

2) Figure 2. It is a bit surprising that the effect of succinate in vitro is much less that observed in vivo (50% vs several logs). Normally, I would assume that delivery of succinate would be easier in vitro and thus better effect. It would be great to test succinate in vitro across airway epithelial cell-lines.

Accordingly, we also assessed the effect of succinate in two additional human lung epithelial cells infected by IAV, *i.e.* bronchial 16hBE14o- cells and alveolar A549 cells. The new appendix Figure S4 confirms that the inhibition of IAV replication (as assessed by a PFU assay) was reduced in a similar range in all airway epithelial cell lines (~61% in BEAS-2B cells, ~61% in 16hBE14o- cells and ~56% in A549 cells, n=3, $p<0.05$ or $p<0.01$).

Both *in vitro* and *in vivo* experiments clearly converge to demonstrate an anti-viral effect of succinate, but the magnitude of the effect is different. Regarding the higher antiviral potency of succinate *in vivo*, one can speculate that this could be related to the multiple mucosal cell subsets (*i.e.* resident and infiltrated leukocytes, parenchyma cells, *etc*) that succinate may regulate *in situ* in addition to the airway epithelial cells. Moreover, direct comparison of *in vitro* and *in vivo* experiments can be tricky because the *in vitro* setting represents a single replication cycle condition in a sole cell type, whereas the *in vivo* setting represents a progressive infection with numerous replication cycles.

3) Figure 4. This figure would benefit from additional data on in vivo virus load. These should include qPCR data for virus mRNA and Western blotting for virus protein on lung samples.

Accordingly, we performed a western-blotting for three IAV proteins (NP, M1 and PA) in lung samples from either IAV-infected mice *vs* IAV-infected and succinate-treated animals. The revised Figure 4 confirms that the viral load (as assessed by the semi-quantification of these IAV proteins) is significantly reduced (>50%, n=4, $p<0.05$). qPCR was not performed due to the clear difference observed at the protein level.

4) Figure 5. Again, related to the timing. The is no observation of difference in mRNA. Even after 24 hours. But here we assume that less virus must be released from the succinate-treated cells. Thus, we must assume that fewer cells are infected from progeny virus. Again, I cannot out-rule

that I am missing point her - but this must be addressed.

As mentioned above (issue 1), our experimental conditions yields a single round of IAV infection. Indeed, in absence of the adequate proteases in the extracellular medium, the released neovirions cannot reinfect non-infected surrounding cells.
Under these experimental settings, we do confirm that there is no difference in viral mRNA in succinate-treated cells, in comparison with non-treated cells ((see the new Figure EV1, panel (c; left side). By contrast, when lung epithelial cells were infected under experimental conditions that support multicycle IAV replication, succinate induces less viral M1 transcripts due to a lower number of reinfecting virus particles (see new Figure EV1, panel (c; right side).

5) Figure 7. The conclusion that NS1 is not involved in the effect of succinate cannot be made without thorough comparison of the effect between the two virus strains. It looks like the reduction by succinate is lower than previously observed. The data here are too few to make that conclusion. Further, is there an effect of succinate on virus replication in the delta-NS1 virus strain?

We agree with the reviewer that a more thorough comparison of the effect of succinate on the replication of wild-type virus *vs* delta-NS1 virus strains would be optimal. However, this type of experiments is rather tricky since NS1-deficiency is sufficient to make IAV very attenuated, leading to a limited viral replication (see also *J Virol*. 2009 83: 6849–6862, DOI: 10.1128/JVI.02323-08).
Nevertheless, we believe that NP (and not NS1) alteration is likely more crucial in the anti-IAV activity of succinate since this metabolite inhibits the replication of the wild-type IAV PR/8/34 (H1N1) strain through a mechanism that involves the nuclear retention of NP but not of NS1 (see Fig. 7a of the manuscript). Moreover, of all IAV proteins, only NP (not NS1) showed lysine succinylation in cells treated with succinate.

6) Figure 8. Many controls to very the NP-vRNA antibody are missing. Uninfected controls for example. Futher, I suggest a simple pull-down experiment to test if the NP-vRNA antibody pulls down less vRNA and NP in succinate treated cells vs controls.

Accordingly, we verified the absence of a fluorescence signal in non-infected cells incubated with either the NP-RNA antibody or the anti-NP antibody. Thus, the revised Fig. 8 clearly shows that the staining related to both antibodies is specific.
Besides, it is important to remind the the NP-vRNA antibody used in our study was developed by R. Webster and R. Webby (St. Jude Children's Research Hospital) and it has been extensively used, controlled and validated by distinct teams (see *PLoS One.* 2016 11: e0149986, doi: 10.1371/journal.pone.0149986 ; *J Virol.* 2011 85: 6117–6126, doi: 10.1128/JVI.00378-11.; *Sci Rep* 2016 6: 29006, doi-org.proxy.insermbiblio.inist.fr/10.1038/srep29006.

Referee #3:

General comment

The finding that succinate inhibits influenza virus infection through succinylation and nuclear retention of the viral nucleoprotein is interesting and important to understand the life cycle of influenza A viruses. It may also provide new ground to develop antivirals. The data provided support the conclusions, but as outlined below, a series of important control experiments are required.

We thank the reviewer #3 for her/his positive opinion and constructive input.

1.) Fig. 3c: From the data provided the inhibition of viral replication by succinate treatment is rather unclear. Two-fold changes are very low for a virus that can replicate up to 10 to 8 PFU/ml. The authors should provide more information by performing single and multitype growth curves. If the viral inhibition is only in the two-fold range it is difficult to believe the EM results shown in Fig. 3d.

We agree with the reviewer #3 (and with reviewer#2 who raised the same issue) that our protocol settings have to be clarified. In fact, lung epithelial cells were challenged with an amount of virus (MOI=1) so that all cells were infected approximately simultaneously and limited to a single cycle of infection. Thus, in absence of the adequate proteases in the extracellular medium, the released neovirions cannot reinfect non-infected surrounding cells.
To meet the reviewer's request, we have tested the effect of succinate on influenza virus-infected lung epithelial cells under experimental conditions (MOI=10-3; presence of TPCK-treated trypsin) that support multicycle replication of the virus. We confirmed and extended our previous findings by showing that succinate impairs influenza virus multiplication even more strongly in multicycle replication condition (~5-fold decrease) than in single replication cycle condition (~2-fold decrease), as evidenced by a plaque-forming unit (PFU) assay (see the new Figure EV1, panels (a) and (b)).

Besides, we agree with the reviewer that transmission electron microscopy (which provides a 2-dimensional picture) could be misleading. Therefore, we replaced our initial picture by another one obtained by scanning electron microscopy (which provides a 3-dimensional image; see revised Figure 3).
Moreover, to help visualizing the inhibitory potency of succinate on influenza virus multiplication, we provide a file containing multiple representative SEM as well as TEM pictures of IAV-infected human lung epithelial cells, treated or not with succinate (see the new Appendix Figure S3)

2.) Fig.4: In Fig. 4d virus is mixed with succinate and then applied to the animal. For a clear interpretation of this in vivo experiment, it is important to know whether mixing the virus with

succinate inactivates the virus. Partial and complete inactivation might have caused the extreme variation in lung titers 4 days post infection. Cell culture experiments (multiple growth curves) can be performed to find out whether succinate inactivates viral particles also directly. If this is the case, the in vivo experiments show in Fig. 4 are not conclusive.

In the first series of our pilot experiments, succinate was not mixed with influenza virus and was administered sequentially in the following order: influenza virus, and then succinate. Under these conditions, succinate enhanced resistance to influenza pneumonia as well. Besides, succinate-induced antiviral activity occurs independently of any contact with the virus, as clearly evidenced in our lung epithelium model. Thus, bronchial epithelial cells were first infected for 4 hours with influenza virus. Then, culture medium containing remaining virus particles was discarded, cells were further washed with PBS and only after, incubated with succinate.

3.) Fig. 4: The K87R mutant virus should show no effect in mice when combined with succinate. Prior in vivo infection experiments the authors should perform growth kinetics (multiple growth curves) whether wt and K87R mutant virus grow similar and whether K87R is affected by succinate treatment.

Using experimental conditions (MOI=10^{-3}; presence of TPCK-treated trypsin) that support multicycle replication of IAV, we did verify that wild-type and K87R mutant virus grow similarly in lung epithelial BEAS-2B cells (Appendix Figure S8).
Regarding the effect of succinate on the growth of NP-K87R influenza virus, we observed *in vitro* that this mutant strain resisted significantly more to this metabokine than the wild-type influenza virus strain (n=6, $p<0.05$; new revised Figure 8g).
Beside, two series of *in vivo* experiments were performed. C57Bl/6 mice were infected intranasally by 100 pfu of either wild-type or K87R IAV strains prior to succinate treatment (also administered intranasally) and animal survival and weight were continuously recorded for three weeks. In fact, those two parameters were not statistically different between the wild-type- and K87R IAV-infected mice. This suggests that *in vivo,* the antiviral effect of succinate is likely the result of not only the impairment of IAV trafficking in lung epithelial cells but also the induction of additional protective signals in lung cell subsets such as resident and infiltrated leukocytes. Determination of the underlying signaling mechanisms and the cells involved in the global antiviral effect of succinate will require further exploration. While this observation is exciting regarding the overall anti-viral effect of succinate, it prevents to make direct comparisons between *in vitro* and *in vivo* experimental set-up. This limitation of our study is stated in the discussion of our revised manuscript (see page 16).
Regardless, this latter result is somehow consistent with the higher antiviral potency of succinate seen *in vivo* compared to its effect measured *in vitro*, as also pinpointed by reviewer #2 (issue 2).

4.) Fig. 6: In panel a the authors show complete retention of NP signal in the cell nucleus after 20 h. This would clearly not be compatible to only 50-60% inhibition in viral growth. Since IF

analysis are often biased, cellular fractionation and subsequent WB analysis should be performed using NP and other antibodies to demonstrate the extend of nuclear retention.

For some unknown reasons, cellular fractionation from IAV-infected cells is often problematic. Thus, we did not succeed to manage this protocol, even though we purchased ready-to-use cell fractionation kits (source: Thermofischer 78833). Because we did not have the expertise to localize viral genomes by fluorescence in situ hybridization (FISH), we opted for an alternative, *i.e*. the detection of NP by flow cytometry using two distinct permeabilization buffers. IAV-infected lung epithelial cells treated or not with succinate were further exposed to True Nuclear™ Transcription Factor buffer that allowed the detection of NP both in the nuclear and cytoplasmic compartments while Cytofix/Cytoperm™ buffer revealed NP only in the cytoplasm (by substraction, one can estimate the amount of NP in the nucleus). Data presented in the Figure EV2 are the mean ± SEM of 4 independent experiments. These flow cytometry results confirm the confocal microscopy data.

5.) Fig. 8: Cell fractionation and WB analysis are required to support the IF data set. Please also provide viral titers at the respective timepoints.

As suggested, we measured the viral titers and found a higher viral load in succinate treated-lung epithelial cells and infected by the mutant NP-K87R influenza virus than in cells infected by the wild-type virus under the same experimental conditions. This suggests that the mutant strain resists significantly more to the antiviral effect of succinate than the wild-type virus strain ($n = 6$, $p<0.01$; see new revised Figure 8g).

6.) The authors speculate that treatment with succinate causes a packaging defect. This can be easily shown.

For the sake of clarity, we reworded as follows our sentence to make it less speculative "*It remains to be determined whether NP succinylation affects the packaging of the viral genome into viral particles*." (see page 16 in the revised manuscript).

Minor points:

1.) Fig. 8a: In this panel it is indicated that the antibody recognizes NP and the viral RNA. Was this experimentally shown or do the authors want to say that the antibody recognizes specifically NP within a vRNP complex?

Yes, the NP-vRNA antibody clone 3/1 recognizes specifically NP within the vRNP complex. It was initially developed by R. Webster and R. Webby (St. Jude Children's Research Hospital) and it has been extensively used, controlled and validated by distinct teams (see *PLoS One.* 2016 11: e0149986, doi: 10.1371/journal.pone.0149986 ; *J Virol.* 2011 85: 6117–6126, doi: 10.1128/JVI.00378-11.; *Sci Rep* 2016 6: 29006, doi-org.proxy.insermbiblio.inist.fr/10.1038/srep29006.

2.) The literature regarding other post translational modifications of IAV proteins and in particular NP is not discussed.

We agree with the reviewer that this information is missing. Accordingly, we added few lines in the discussion section (see page 15 in the revised manuscript) reminding that IAV particles and viral components interact with the host cellular machinery, including the post-translational modifications (PTMs) components. The most common PTMs include phosphorylation, ubiquitination, SUMOylation, acetylation, methylation, NEDDylation, and glycosylation. Many PTMs foster influenza virus infection, whereas others can contribute to antiviral defense (Hu *et al.* Front. Microbiol., 2020; https://doi.org/10.3389/fmicb.2020.517461).

Dear Dr Si-Tahar,

Thank you for submitting your revised manuscript (EMBOJ-2021-108306R) to The EMBO Journal. Your amended study was sent back to the two referees for re-evaluation, and we have received comments from both of them, which I enclose below.

As you will see, referee #2 stated that the issues raised have been adequately addressed and s/he is now broadly in favour of publication. Reviewer #3 finds the study to be improved, but also points to remaining ambiguities related to the mechanism of inhibition and antiviral activity of succinate.

We have carefully considered the experts' input and concluded that while referee #3's remaining point is well taken, we are overall able to proceed with this work, pending a minor final revision. I am thus pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining point of referee #3 carefully, and address these by introducing caveats in the discussion of the results where appropriate.

In addition, we need you to take care of a minor issue related to data documentation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might have seen on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Please add http links to the data sets listed in the data availability section and remove the referee information.

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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (14th Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2

I think the authors have adequately addressed my concerns and and suggestions.
I fully recommend publication without delay.

Referee #3

The mechanism of succinate inhibition is still not clear. Influenza is replicating in the cell nucleus and around 5-7 h post infection vRNPs are exported to the cytoplasm. Massive budding of viral particles occurs 7-20 h post infection. Retention of NP signal in the cell nucleus 20 hours post infection is not compatible with the observed small effect of succinate on viral replication. In addition, the K87R mutant virus does not provide the clear cut picture as hoped. Especially in vivo the authors must acknowledge that other effects can occur. However, there are technical issues that complicate the analysis and thus defining the mode of action could be quite complicated.

Dear Dr. Klimmeck,

We thank you and the reviewers very much for your positive evaluation of our manuscript entitled: ***"Host succinate inhibits influenza virus infection through succinylation and nuclear retention of the viral nucleoprotein"***

Please find below our point-by-point response to your last requests. The corresponding changes were made in the revised manuscript (highlighted in yellow).

Once again, we thank you for your interest in our work and for your thoughtful comments and suggestions as well as those from the reviewers.

- *Editor: Please consider the remaining point of referee #3 carefully, and address these by introducing caveats in the discussion of the results where appropriate.*
- *Referee #3: The mechanism of succinate inhibition is still not clear (…) Especially in vivo the authors must acknowledge that other effects can occur. However, there are technical issues that complicate the analysis and thus defining the mode of action could be quite complicated.*

As requested by the reviewer #3, we acknowledge that "*in vivo*, the antiviral effect of succinate is likely the result of not only the impairment of influenza virus trafficking in lung epithelial cells but also the involvement of additional mechanisms, e.g. inhibitory effects in other lung cell subsets such as resident and/or infiltrated leukocytes" (see lines 391-396 of the revised manuscript)

- *Please add http links to the data sets listed in the data availability section and remove the referee information.*

This has been modified accordingly (see lines 684-686 of the revised manuscript)

- *I would appreciate if you could provide this (synopsis) figure and the bullet points.*

We have included in the revised documents, a model figure and a text with bullet points that summarize our study.

Dear Dr Si-Tahar,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in 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.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you for this contribution to The EMBO Journal and congratulations on a successful publication!

Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
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EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	SUPPLEMENTARY TABLE: List of all reagents and resources used

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	SUPPLEMENTARY TABLE: List of all reagents and resources used Materials and Methods (Cell culture)
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	All cell lines are mycoplasma free (one test per month)

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	SUPPLEMENTARY TABLE: List of all reagents and resources used Materials and Methods (Animal infection and fluid collection)
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	SUPPLEMENTARY TABLE: List of all reagents and resources used Materials and Methods (Viruses and reverse genetics)

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Materials and Methods (Tracheal Aspirate collection)

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Methods
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

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Methods
Studies involving **human participants**: 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.	Yes	Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Methods
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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	
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 availibility
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?	Yes	Methods
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	