EMBO reports

Single-cell transcriptomics identifies Gadd45b as a regulator of herpesvirus-reactivating neurons

Hui-Lan Hu, Kalanghad Srinivas, Shuoshuo Wang, Moses Chao, Timothee Lionnet, Ian Mohr, Angus Wilson, Daniel Depledge, and Tony Huang
DOI: 10.15252/embr.202153543

Corresponding author(s): Tony Huang (tony.huang@nyumc.org) , Angus Wilson (wilsoa02@med.nyu.edu), Daniel Depledge (Depledge.Daniel@mh-hannover.de)

Review Timeline:

Submission Date:	29th Jun 21
Editorial Decision:	15th Jul 21
Revision Received:	7th Oct 21
Editorial Decision:	7th Nov 21
Revision Received:	10th Nov 21
Accepted:	11th Nov 21

Editor: Achim Breiling

Transaction Report:

(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. Huang,

Thank you for the transfer of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

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

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

See also our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses 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://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an

appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

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: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that, where applicable, the number "n" for how many independent experiments were performed and the type of replicate (biological or technical), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

9) Please note our new reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) Please add up to 5 key words to the title page, a conflict-of-interest statement and a paragraph detailing the author contribution to the manuscript and order the sections like this (using this nomenclature):
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods -Data availability section - Acknowledgements - Author contributions - Conflict of interest statement - References - Figure legends - Expanded View Figure legends.

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This group has been instrumental in elucidating some of the molecular details regarding the reactivation of HSV from latency in neuronal cells. This study goes on to utilize single cell RNA sequencing and single molecule FISH to identify host transcriptome changes during latency and reactivation. They provide data that further their two-step model for reactivation, and identify cellular genes, Gadd45 in particular, that are induced during latent infection. It was found that Gadd45 suppressed one step in the HSV reactivation process. Its presence in the nucleus correlated with reduced late HSV gene expression. They also found that it reduced the abundance of ICP4, which is need for late gene transcription. An attractive model is presented that predicts whether reactivation is productive or aborted depending on the subcellular localization of Gadd45. This is an elegant study. The conclusions are well supported by the data and the manuscript is well written. It will be of great interest to the field and general audiences as well. There are just some few minor comments:
1. Probably the most important is that it is often stated in the text (including the abstract) that Gadd45 inhibits ICP4. In fact the abundance of ICP4 is reduced. Is it inhibiting its expression at some level or its stability? There is no data to show that it inhibits its activity, which the phrase "inhibits ICP4" implies. Please rephrase.
2. There is a line, "....viral DNA replication dependent proteins are only made after 48 hrs as measured by fluorescent detection of the true late viral gene product, GFP-Us11 (Figure 2B)." They only measured one. Please rephrase.
3. Can one really be so sure that the effects of Gadd45 on ICP4 accumulation only affect late transcription? Couldn't be that late transcription is the most sensitive because several prior processes are need to cooperated to direct abundant late gene expression.
4. I'm sure glad there were not many issues with this manuscript. Not having page or line numbers would have made for a difficult review.

Referee #2:

This study by Hu et al. combines the use of two technologies, single-cell RNA sequencing (scRNA-seq) and single-molecule sensitivity fluorescent in situ hybridization (smFISH) to decipher the interactions between primary rat superior cervical ganglia (SCG) neurons and latent/reactivating HSV-1. The model used consists in cultured primary neurons isolated from dissociated superior cervical ganglia of embryonic rats infected by HSV-1. HSV-1 latency is established using acyclovir (ACV), a potent inhibitor of viral replication, and reactivation using LY294002 an inhibitor of the PI-3K, known to induce reactivation in this specific model. By analyzing single-cell host cell transcriptome changes upon infection the authors identified the stress sensor Growth arrest and DNA damage-inducible 45 beta (Gadd45b) as a critical antiviral host factor that regulates HSV-1 reactivation events in a subpopulation of primary neurons. They demonstrate that Gadd45b suppresses viral late gene expression through the inhibition of the synthesis of the viral transcription factor ICP4. They also show that successful reactivation relies on the differential nucleo-to-cytoplasmic relocation of Gadd45b by an unknown viral-infection associated mechanism.
The study is most interesting, the data are clean and the conclusions are in accordance with the data provided. The reviewer has concerns regarding some experimental issues that should be raised to improve the quality of the study.

A. Major items to be addressed
General :
1) To reviewer's view there is a possible confusion for the reader in the understanding of the Gadd45b protein pattern in latently infected neurons between cultures not treated and treated with LY (figure 5A vs 5E). The reviewer understands from the reading of the text that after LY stimulus, 9% of neurons that do not reactivate (EDU-) show punctate Gadd45b and those neurons are all LAT+ (Figure 5E). To reviewer's view one important missing information is the percentage of LAT+ neurons showing Gadd45b in puncta following LY treatment. In other words, what is the percentage of LAT+ neurons showing nuclear punctate vs diffuse Gadd45b pattern after LY treatment? This should be clarified.

2) P9 the authors mention : *" As a control, we observed similar pan-nuclear staining for Gadd45b, but did not observe any nuclear foci/puncta staining pattern in LY-treated, uninfected neurons (Figure 5A-B). ».* This means that nuclear puncta for Gadd45b appears only if neuron populations have been initially infected. Given that the study does not mention the percentage of LAT+ neurons with Gadd45b puncta pattern, one could conclude that the puncta Gadd45b pattern upon LY addition could be due to initial priming of the neuron population by a paracrine or autocrine signal, such as IFN-I-associated response, following HSV-1 infection, if not by the ACV treatment itself? It is to reviewer's view particularly important to clarify this aspect given that the study attributes to Gadd45b a restrictive antiviral activity. The authors should test the potential contribution of 1) IFN-I pre-priming and 2) AVC addition, before LY treatment, on Gadd45b pattern in non-infected neuronal cultures, to show if the puncta pattern of Gadd45 is indeed associated to the infected cells rather than a consequence of an antiviral response to the infection or simply to the ACV treatment.

Specific
1) Figure 4A, 4G, 4H. There is an anti-parallel correlation between Gadd45b and UL36 smFISH signal intensity and/or true late GFP-US11 signal detection. The initial increase of the Gadd45b RNA signal is seemingly not due to the PI-3K pathway inactivation (Figure 4B and D). Therefore, it is inferred that viral reactivation induces the increase of Gadd45b RNA amount at

the initial stage of the reactivation followed by a decrease concomitantly with the increase in UL36 RNA and/or US11 detection (Lg2 genes), and presumably progress into the replication phase of the lytic cycle. If true, then blocking viral replication during the reactivation process by addition of PAA should prevent the decrease of Gadd45b RNA and would be a good indicator of an active viral replication-associated process implicated in the control of Gadd45b RNA production. RT-qPCR to quantify at least Gadd45b mRNA should be provided following LY reactivation and in the presence of PAA. This will definitively prove that the Gadd45b smFISH signal decrease correlates with a decrease in Gadd45b mRNA amount in cells expressing viral replication-associated lytic genes. Even better, but to reviewer's view not compulsory for the general understanding of the study, would be to provide scRNA transcriptome in the condition of reactivation induced in the presence of PAA. This would enable to specifically compare the behavior of the Gadd45b transcripts compared to the 19 other cellular mRNAs found up-regulated in the reactivating SCG neurons (Figure 3b).

B. Other

General

1) In general the reviewer has some semantic and experimental concerns about the localization of the Gadd45b in the so called HSV-1 latently-infected neurons. Indeed, HSV-1 latency in neurons is defined in the study by the sole expression of the latency associated transcript (LAT) and not by the visualization of the viral genomes.
As an example P9 the authors state : *« ...we found that Gadd45b was present in both nuclear and cytoplasmic compartments in uninfected and **HSV-1 latently-infected neurons** (Figure 5A-B)".* However, page 6, the authors mention that about 60% of the SCG neurons show detectable LAT expression (Figure 1D). The authors also mention the fact that some infected neurons may possess insufficient amount of detectable LAT, which supposes that some neurons are latently infected but are LAT-. This is a well-known aspect of latently infected mouse and human neurons in vivo at least in TG (Mehta et al, 1995; Sawtell et al, 1998; Wang et al, 2005; Catez et al, 2012). To reviewer's view it is thus difficult to specifically and individually refer to HSV-1 latently-infected neurons in the case of neurons that are LAT-, without showing the presence of viral DNA. Therefore, caution should be used in the description of the Gadd45b localization in latently infected neurons, if the latency state refers only to those neurons which are LAT+. Authors should probably rather refer to latently infected neuron culture/population.

2) Consequently and similarly to the previous comment, the sentence : *"This suggests that the Gadd45b nuclear foci/puncta staining pattern coincides with **latently-infected neurons** that fail to reactivate »* should be changed in :*"This suggests that the Gadd45b nuclear foci/puncta staining pattern coincides with latently-infected neurons population in which some neurons fail to reactivate following LY treatment»* as without the detection of the presence of viral genomes in the EDU- and LAT- neurons it is difficult to predict that those neurons are indeed infected.

3) On a more technical aspect, the reviewer does not understand how smFISH can discriminate between pre-mRNA and mRNA ? The authors mention the detection of Gadd45b mRNA by smFISH, but how could they be sure that they detect only the mRNA and not the pre-mRNA when the smFISH signal is nuclear? It is important given the differential location of the Gadd45b RNA detection (nucleus vs cytoplasm) in latent/reactivating neurons (Figure 4A, 4B).

4) Given the complexity of the different signals corresponding to Gadd45b RNA and/or protein in different situations the authors should provide a table with the different mRNA/protein Gadd45b patterns and the different states of viral latency/reactivation (LAT-/+, UL30/36 -/+, EDU-/+, ICP4-/+, US11-/+).

Specific

1) Figure 1D : Detection of LAT by smFISH. Can smFISH discriminate between LAT 8.3kb (minor LAT) and LAT introns (major LAT)? It has been shown that minor LAT could be detected by ISH or classical RNA FISH under the form of large spots in latently infected mouse TG neurons, provided that a probe directed against the 5' minor LAT region is used (Arthur et al., 1993; Catez et al. 2012). Given the high sensitivity of smFISH that allows to detect low amount of LAT (Figure 1D, LAT (low)), one would expect to visualize the spotty pattern of the minor LAT even if the smFISH probes are designed in the intron region. Could the authors comment on this aspect of the non-detection of the minor LAT? Does it exist an intermediate labelling between the LAT (low) and LAT (high) patterns? Alternatively if the authors came across such "large spots" patterns of LAT (for example under the form of grouped smFISH LAT signals), just like what is detected for the nuclear smFISH signals for UL30, UL36, ICP27 (Figure 2C), it would be interesting to show it.

2) Figure 2C. smFISH detects viral transcripts UL36 co-localizing with UL30 or ICP27 RNAs in reactivating SCG neurons. The authors comment on the possibility that those viral transcripts could be detected on the same episomal viral genomes (page 7). Would it be conceivable to combine smFISH with DNA-FISH to prove this statement?

3) P9 the authors state : *"Thus, these results demonstrate that cellular expression of select Gadd45 isoforms, Gadd45b and Gadd45g, **during conditions of HSV-1 reactivation** in neurons antagonizes the HSV-1 late gene expression program »* As far as the reviewer understands Figure 6A and B the experiments are performed without addition of LY, therefore not in conditions of HSV-1 reactivation. So, mentioning "during conditions of HSV-1 reactivation" is misleading because, according to the data, the sole depletion of Gadd45b by a shRNA is enough to induce reactivation without addition of LY. Please clarify.

4) Figure 6 : Please provide if possible a WB for the detection of endogenous Gadd45b protein following shRNA treatments.

5) P11 : "... Gadd45b also suppressed Mirin-induced..." change by "... Mirin-induced HSV-1 reactivation".

6) Figure 6 G : Please provide quantification of ICP4 signals +/- Gadd45bMycFlag as tubulin signal is not homogenous

7) How could downregulation of ICP4 by Gadd45b impact on HSV-1 reactivation and not on productive lytic infection? This should be discussed further.

8) In the discussion P11 the authors state : *"By monitoring a limited set of viral transcripts, we and others have described a unique biphasic program of viral gene transcription through which the latent viral genomes transition from a transcriptionally repressed state into active replication culminating in the production of new infectious progeny »*. The biphasic program of reactivation has been observed in models of HSV-1 latency obtained using ACV. However, it is likely that the use of ACV does not recapitulate the real process of establishment of HSV latency in neurons in vivo, at least from what we know in the mouse model. Studies, among other by Efstathiou's lab, using Cre-lox system combined to Rosa mice nicely showed that latently infected neurons hardly show historic of E, Lg1 or Lg2 genes expression (Proenca et al, 2008), which should be the case using ACV at least for E and Lg1 genes. This ACV-specific model of HSV-1 latency establishment should be specified whenever statements putting forwards molecular aspects of HSV-1 reactivation are made.

Catez F, Picard C, Held K, Gross S, Rousseau A, Theil D, Sawtell N, Labetoulle M & Lomonte P (2012) HSV-1 Genome Subnuclear Positioning and Associations with Host-Cell PML-NBs and Centromeres Regulate LAT Locus Transcription during Latency in Neurons. PLoS Pathog 8: e1002852

Mehta A, Maggioncalda J, Bagasra O, Thikkavarapu S, Saikumari P, Valyi-Nagy T, Fraser NW & Block TM (1995) In situ DNA PCR and RNA hybridization detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice. Virology 206: 633-640

Proenca JT, Coleman HM, Connor V, Winton DJ & Efstathiou S (2008) A historical analysis of herpes simplex virus promoter activation in vivo reveals distinct populations of latently infected neurones. J Gen Virol 89: 2965-2974

Sawtell NM, Poon DK, Tansky CS & Thompson RL (1998) The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. Journal of virology 72: 5343-5350

Wang K, Lau TY, Morales M, Mont EK & Straus SE (2005) Laser-capture microdissection: refining estimates of the quantity and distribution of latent herpes simplex virus 1 and varicella-zoster virus DNA in human trigeminal Ganglia at the single-cell level. Journal of virology 79: 14079-14087

Referee #3:

In this manuscript from Hu et al, the authors use a combination of different single cell techniques to identify both a positive and negative regulator of Herpes Simplex Virus 1 reactivation. This is a well-executed study that will have a major impact on the field. The authors focus mainly on the negative regulator, Gadd45b and show that it is induced in neurons infected with HSV in response to a stimulus of reactivation (LY294002). Importantly, the induction was only detected in infected neurons and correlated with reduced UL36 gene expression (encodes a late viral protein) and reduced Us11-GFP positive neurons. Furthermore, expression of Gadd45b and Gadd45g inhibited HSV reactivation whereas Gadd45b knockdown resulted in spontaneous reactivation. This is a novel observation and as yet no role for Gadd45b/g in HSV reactivation has been identified. In addition, identification of a negative regulator that acts downstream of the initiation of gene expression is important as it highlights the potential for abortive reactivation. The authors make a number of additional observations that are important for the field including a thorough characterization at the single cell level of viral lytic mRNAs and the potential for expression from one or multiple viral genomes. The one weakness in the paper is the role for ICP4. The conclusion that Gadd45b suppresses viral late gene expression through the inhibition of the viral transcription factor, ICP4, is not well supported by the data.

1. The data on ICP4 is correlative. The observations are 1) that a lack of detectible ICP4 correlates with Gadd45b puncta (but not nuclear staining) 2) that ICP4 mRNA levels are reduced at 72h post-LY with Gadd54b expression. However, expression of other mRNAs including another IE gene ICP27 and early gene UL30 are also reduced with Gadd45 expression, as is viral DNA load. Given that the authors show that as reactivation proceeds (and is more successful) the levels of viral mRNAs/cell increase, iss it therefore possible that Gadd45b suppresses this general increase across the genome? It is surprising that the authors did not extend their smFISH assays from figure 2 to examine the consequence of Gadd45b expression on changes in viral mRNA levels over time and this would help strengthen the paper and address the potential for Gadd45b to prevent the transition to productive reactivation.

2. A continued increase in viral mRNA between 48 and 72 hours could be indicative of cell spread. The authors should show that

the stock of WAY150138 used is active in preventing cell-to-cell spread.

3. The authors interpret the data in Figure 2 as viral DNA replication dependent proteins are only made after 48 hours based on the detection of Us11-GFP compared to smFISH for ICP27, UL30 and UL36. The authors do not have enough data to make this conclusion. First, the smFISH assay is likely more sensitive than immunofluorescence and this may represent a limit of detection of viral protein. They also do not look at Us11 mRNA nor ICP27, UL30 or UL36 protein levels. It is also possible that GFP-detection is less sensitive. This is an important consideration in the HSV field and therefore the caveats need more thoroughly addressing. Immunofluorescence for additional viral proteins would strengthen the manuscript.

4. A main conclusion of the paper on the role for Gadd45b inhibiting reactivation uses lentivirus mediated delivery of a transgene. The additional of lentivirus alone could have an effect on the neuronal cultures (the authors even see some spontaneous reactivation). The neurons also need to be transduced with a negative control lentivirus.

5. The EdU experiments permit the identification or viral DNA replication and are a great addition to the manuscript. However, EdU can have toxic effects. Therefore, the authors should show that the addition of EdU does not alter the subcellular localization of Gadd45b.

Minor points:

1. Multiple figures throughout the manuscript lack statistics. It would also be beneficial if the authors plotted individual biological replicates.
2. The authors do not mention in the methods how long EdU pulsing was carried out.
3. In figure 2D it would be clearer if the y-axis were labelled as % positive neurons that are double positive.
4. It is not clear why Figure S3C is ICP27 mRNA were as the other panels show GFP-positive neurons.

Re: EMBOR-2021-53543-T

We thank all three reviewers for their time in providing useful feedback on our manuscript. Point-by-point responses are provide below (reviewer comments in italics / our rebuttal in blue).New or amended data figures are cited where relevant:

Referee #1:

This group has been instrumental in elucidating some of the molecular details regarding the reactivation of HSV from latency in neuronal cells. This study goes on to utilize single cell RNA sequencing and single molecule FISH to identify host transcriptome changes during latency and reactivation. They provide data that further their two-step model for reactivation, and identify cellular genes, Gadd45 in particular, that are induced during latent infection. It was found that Gadd45 suppressed one step in the HSV reactivation process. Its presence in the nucleus correlated with reduced late HSV gene expression. They also found that it reduced the abundance of ICP4, which is need for late gene transcription. An attractive model is presented that predicts whether reactivation is productive or aborted depending on the subcellular localization of Gadd45. This is an elegant study. The conclusions are well supported by the data and the manuscript is well written. It will be of great interest to the field and general audiences as well.

We appreciate the Reviewer's enthusiasm for our study.

There are just some few minor comments:
1. Probably the most important is that it is often stated in the text (including the abstract) that Gadd45 inhibits ICP4. In fact the abundance of ICP4 is reduced. Is it inhibiting its expression at some level or its stability? There is no data to show that it inhibits its activity, which the phrase "inhibits ICP4" implies. Please rephrase.

We agree that we did not provide evidence to show direct inhibition of ICP4 function by Gadd45b and have now reworded this in the text and abstract accordingly.

2. There is a line, "....viral DNA replication dependent proteins are only made after 48 hrs as measured by fluorescent detection of the true late viral gene product, GFP-Us11 (Figure 2B)." They only measured one. Please rephrase.

We have rephrased this point in the results section describing Figure 2B.

3. Can one really be so sure that the effects of Gadd45 on ICP4 accumulation only affect late transcription? Couldn't be that late transcription is the most sensitive because several prior processes are need to cooperated to direct abundant late gene expression.

Yes, we agree with the reviewer that the effect of Gadd45b on ICP4 accumulation could affect other upstream processes that may indirectly alter late viral transcription, we have softened this statement throughout the text.

4. I'm sure glad there were not many issues with this manuscript. Not having page or line numbers would have made for a difficult review.

We apologize for this oversight.

Referee #2:

This study by Hu et al. combines the use of two technologies, single-cell RNA sequencing (scRNA-seq) and single-molecule sensitivity fluorescent in situ hybridization (smFISH) to decipher the interactions between primary rat superior cervical ganglia (SCG) neurons and latent/reactivating HSV-1. The model used consists in cultured primary neurons isolated from dissociated superior cervical ganglia of embryonic rats infected by HSV-1. HSV-1 latency is established using acyclovir (ACV), a potent inhibitor of viral replication, and reactivation using LY294002 an inhibitor of the PI-3K, known to induce reactivation in this specific model. By analyzing single-cell host cell transcriptome changes upon infection the authors identified the stress sensor Growth arrest and DNA damage-inducible 45 beta (Gadd45b) as a critical antiviral host factor that regulates HSV-1 reactivation events in a subpopulation of primary neurons. They demonstrate that Gadd45b suppresses viral late gene expression through the inhibition of the synthesis of the viral transcription factor ICP4. They also show that successful reactivation relies on the differential nucleo-to-cytoplasmic relocation of Gadd45b by an unknown viral-infection associated mechanism. The study is most interesting, the data are clean and the conclusions are in accordance with the data provided. The reviewer has concerns regarding some experimental issues that should be raised to improve the quality of the study.

We thank the Reviewer's positive comments pertaining to our study.

A. Major items to be addressed General :1) To reviewer's view there is a possible confusion for the reader in the understanding of the Gadd45b protein pattern in latently infected neurons between cultures not treated and treated with LY (figure 5A vs 5E). The reviewer understands from the reading of the text that after LY stimulus, 9% of neurons that do not reactivate (EDU-) show punctate Gadd45b and those neurons are all LAT+ (Figure 5E). To reviewer's view one important missing information is the percentage of LAT+ neurons showing Gadd45b in puncta following LY treatment. In other words, what is the percentage of LAT+ neurons showing nuclear punctate vs diffuse Gadd45b pattern after LY treatment? This should be clarified.

The percentage of LAT+ neurons showing Gadd45b nuclear puncta staining is ~18%, and ~40% showing diffuse cytoplasmic staining. This has now been clarified in the text (page 9).

2) P9 the authors mention :*" As a control, we observed similar pan-nuclear staining for Gadd45b, but did not observe any nuclear foci/puncta staining pattern in LY-treated, uninfected neurons (Figure 5A-B). ».* This means that nuclear puncta for Gadd45b appears only if neuron populations have been initially infected. Given that the study does not mention the percentage of LAT+ neurons with Gadd45b puncta pattern, one could conclude that the puncta Gadd45b pattern upon LY addition could be due to initial priming of the neuron population by a paracrine or autocrine signal, such as IFN-I-associated response, following HSV-1 infection, if not by the ACV treatment itself? It is to reviewer's view particularly important to clarify this aspect given that the study attributes to Gadd45b a restrictive antiviral activity. The authors should test the potential contribution of 1) IFN-I pre-priming and 2) AVC addition, before LY treatment, on Gadd45b pattern in non-infected neuronal cultures, to show if the puncta pattern of Gadd45 is indeed associated to the infected cells rather than a consequence of an antiviral response to the infection or simply to the ACV treatment.

We tested the potential contribution of ACV treatment in affecting Gadd45b localization pattern in non-infected neuronal cultures. This has now been included in the **new Figure EV2C** (compared

to Figure 5C), showing that Gadd45b staining pattern looks similar to mock treated non-infected neuronal cultures (LY treatment causes nuclear accumulation Gadd45b, regardless of the presence of virus). Even with ACV treatment, there was ~0% of neurons showing Gadd45b nuclear puncta staining. This has now been clarified in the text (page 9). We did not test whether IFN-I treatment would affect Gadd45b staining pattern. The idea of IFN-I-associated host response that primes the neuronal population by a paracrine or autocrine signal is very intriguing, but we feel this is beyond the scope of this current study.

Specific:
1) Figure 4A, 4G, 4H. There is an anti-parallel correlation between Gadd45b and UL36 smFISH signal intensity and/or true late GFP-US11 signal detection. The initial increase of the Gadd45b RNA signal is seemingly not due to the PI-3K pathway inactivation (Figure 4B and D). Therefore, it is inferred that viral reactivation induces the increase of Gadd45b RNA amount at the initial stage of the reactivation followed by a decrease concomitantly with the increase in UL36 RNA and/or US11 detection (Lg2 genes), and presumably progress into the replication phase of the lytic cycle. If true, then blocking viral replication during the reactivation process by addition of PAA should prevent the decrease of Gadd45b RNA and would be a good indicator of an active viral replication-associated process implicated in the control of Gadd45b RNA production. RT-qPCR to quantify at least Gadd45b mRNA should be provided following LY reactivation and in the presence of PAA. This will definitively prove that the Gadd45b smFISH signal decrease correlates with a decrease in Gadd45b mRNA amount in cells expressing viral replication-associated lytic genes. Even better, but to reviewer's view not compulsory for the general understanding of the study, would be to provide scRNA transcriptome in the condition of reactivation induced in the presence of PAA. This would enable to specifically compare the behavior of the Gadd45b transcripts compared to the 19 other cellular mRNAs found up-regulated in the reactivating SCG neurons (Figure 3b).

We apologize for being unclear in our description of the relationships between the smFISH signals for Gadd45b and UL36 (and GFP-Us11 fluorescence). We don't think the anti-parallel correlation between Gadd45b and UL36 signals infers a timed progression of different viral reactivation stage, instead we think the difference reflects the inherent heterogeneity of the latently-infected cultured neurons. Nevertheless, we performed the PAA (viral DNA polymerase inhibitor) experiment as requested by the Reviewer (**new Figure 5A**). Interestingly, we found that PAA treatment was capable of inhibiting LY-induced Gadd45b mRNA expression in latently-infected SCG neurons. This suggests that Gadd45b mRNA expression is upregulated in response to viral DNA synthesis. Also, Gadd45b mRNA expression levels were higher at 72 hrs than at 48 hrs (**new Figure 5A**), indicating that Gadd45b mRNA levels did not initially increase then decrease due to a block in Gadd45b transcription by some viral gene products.

*B. Other General 1) In general the reviewer has some semantic and experimental concerns about the localization of the Gadd45b in the so called HSV-1 latently-infected neurons. Indeed, HSV-1 latency in neurons is defined in the study by the sole expression of the latency associated transcript (LAT) and not by the visualization of the viral genomes. As an example P9 the authors state : « ...we found that Gadd45b was present in both nuclear and cytoplasmic compartments in uninfected and **HSV-1 latently-infected neurons** (Figure 5A-B)". However, page 6, the authors mention that about 60% of the SCG neurons show detectable LAT expression (Figure 1D). The authors also mention the fact that some infected neurons may possess insufficient amount of detectable LAT, which supposes that some neurons are latently infected but are LAT-. This is a well-known aspect of latently infected mouse and human neurons in vivo at least in TG (Mehta et al, 1995; Sawtell et al, 1998; Wang et al, 2005; Catez et al, 2012). To reviewer's view it is thus difficult to specifically and individually refer to HSV-1 latently-infected neurons in the case of*

neurons that are LAT-, without showing the presence of viral DNA. Therefore, caution should be used in the description of the Gadd45b localization in latently infected neurons, if the latency state refers only to those neurons which are LAT+. Authors should probably rather refer to latently infected neuron culture/population.

Thank you for this point, we have now clarified “latently-infected neurons” to “latently-infected cultured neurons/population” throughout the text.

2) Consequently and similarly to the previous comment, the sentence : "This suggests that the Gadd45b nuclear foci/puncta staining pattern coincides with ***latently-infected neurons*** *that fail to reactivate » should be changed in : "This suggests that the Gadd45b nuclear foci/puncta staining pattern coincides with latently-infected neurons population in which some neurons fail to reactivate following LY treatment» as without the detection of the presence of viral genomes in the EDU- and LAT- neurons it is difficult to predict that those neurons are indeed infected.*

Noted, and now clarified as suggested by the Reviewer (page 9).

3) On a more technical aspect, the reviewer does not understand how smFISH can discriminate between pre-mRNA and mRNA ? The authors mention the detection of Gadd45b mRNA by smFISH, but how could they be sure that they detect only the mRNA and not the pre-mRNA when the smFISH signal is nuclear? It is important given the differential location of the Gadd45b RNA detection (nucleus vs cytoplasm) in latent/reactivating neurons (Figure 4A, 4B).

In general, the mRNA molecules are significantly more abundant and stable than pre-mRNAs which are rapidly processed or turned over. It is thus a sensible assumption that the smFISH signal is primarily detecting mRNAs.

4) Given the complexity of the different signals corresponding to Gadd45b RNA and/or protein in different situations the authors should provide a table with the different mRNA/protein Gadd45b patterns and the different states of viral latency/reactivation (LAT-/+, UL30/36 -/+, EDU-/+, ICP4-/+, US11-/+).

We recognize that by following multiple viral marker across different experimental conditions the data are not simple. Hopefully the clarifications described above will makes this easier for readers to navigate without the risk of over-simplifying the information by translating into tabular form.

Specific
1) Figure 1D : Detection of LAT by smFISH. Can smFISH discriminate between LAT 8.3kb (minor LAT) and LAT introns (major LAT)? It has been shown that minor LAT could be detected by ISH or classical RNA FISH under the form of large spots in latently infected mouse TG neurons, provided that a probe directed against the 5' minor LAT region is used (Arthur et al., 1993; Catez et al. 2012). Given the high sensitivity of smFISH that allows to detect low amount of LAT (Figure 1D, LAT (low)), one would expect to visualize the spotty pattern of the minor LAT even if the smFISH probes are designed in the intron region. Could the authors comment on this aspect of the non-detection of the minor LAT? Does it exist an intermediate labelling between the LAT (low) and LAT (high) patterns? Alternatively if the authors came across such "large spots" patterns of LAT (for example under the form of grouped smFISH LAT signals), just like what is detected for the nuclear smFISH signals for UL30, UL36, ICP27 (Figure 2C), it would be interesting to show it.

Yes, the synthesis and accumulation of RNAs originating from the LAT locus would be very interesting to look at in our model but at this time, it is unclear how this would speak to the focus of this particular study. For our purposes, smFISH detection of LAT RNA is used primarily as a marker of viral infection. The LAT-specific oligonucleotides used in the smFISH probes are listed in the Appendix Table S5.

2) Figure 2C. smFISH detects viral transcripts UL36 co-localizing with UL30 or ICP27 RNAs in reactivating SCG neurons. The authors comment on the possibility that those viral transcripts could be detected on the same episomal viral genomes (page 7). Would it be conceivable to combine smFISH with DNA-FISH to prove this statement?

We currently don't know if the different viral transcripts are detected in proximity to the same episomal viral genome or not, but it is striking that some of the nuclear smFISH signal spots do appear to overlap in our images. We have not yet been successful in combining the smFISH and DNA-FISH techniques to explore this more directly but hope to revisit this technically difficult analysis in our future studies.

3) P9 the authors state : "Thus, these results demonstrate that cellular expression of select Gadd45 isoforms, Gadd45b and Gadd45g, ***during conditions of HSV-1 reactivation*** *in neurons antagonizes the HSV-1 late gene expression program ». As far as the reviewer understands Figure 6A and B the experiments are performed without addition of LY, therefore not in conditions of HSV-1 reactivation. So, mentioning "during conditions of HSV-1 reactivation" is misleading because, according to the data, the sole depletion of Gadd45b by a shRNA is enough to induce reactivation without addition of LY. Please clarify.*

We apologize for this confusion, we have now clarified this statement (page 10), "Thus, these results suggest that cellular expression of Gadd45b (and likely Gadd45g) during the virus latent state in neurons antagonizes the HSV-1 late gene expression program in order to prevent spontaneous HSV-1 reactivation."

4) Figure 6 : Please provide if possible a WB for the detection of endogenous Gadd45b protein following shRNA treatments.

Unfortunately, none of the commercially available antibodies against Gadd45b work well (in our hands) to detect endogenous Gadd45b protein in Rat neurons. However, we have provided data showing that Gadd45b mRNA levels are significantly reduced after Gadd45b shRNA knockdown treatments (Figure 6A), and for the other Gadd45 forms (Figure EV3A and B).

5) P11 : "... Gadd45b also suppressed Mirin-induced..." change by "... Mirin-induced HSV-1 reactivation".

Noted.

6) Figure 6 G : Please provide quantification of ICP4 signals +/- Gadd45bMycFlag as tubulin signal is not homogenous

In the **new Figure 6G**, we provide quantitation of the ICP4 western blot signals (performed in triplicate), showing that steady-state ICP4 protein level are reduced roughly two-fold at LY72 in Gadd45b-expressing cells (compared to mock control).

7) How could downregulation of ICP4 by Gadd45b impact on HSV-1 reactivation and not on productive lytic infection? This should be discussed further.

We have now added this section in the discussion (see page 16).

8) In the discussion P11 the authors state : "By monitoring a limited set of viral transcripts, we and others have described a unique biphasic program of viral gene transcription through which the latent viral genomes transition from a transcriptionally repressed state into active replication culminating in the production of new infectious progeny ». The biphasic program of reactivation has been observed in models of HSV-1 latency obtained using ACV. However, it is likely that the use of ACV does not recapitulate the real process of establishment of HSV latency in neurons in vivo, at least from what we know in the mouse model. Studies, among other by Efstathiou's lab, using Cre-lox system combined to Rosa mice nicely showed that latently infected neurons hardly show historic of E, Lg1 or Lg2 genes expression (Proenca et al, 2008), which should be the case using ACV at least for E and Lg1 genes. This ACV-specific model of HSV-1 latency establishment should be specified whenever statements putting forwards molecular aspects of HSV-1 reactivation are made.

We have now provided a statement about the use of acyclovir in our *in vitro* latency model to suppress viral replication during the initial infection stage in Figure 1 (page 5). The use of ACV in our experiments is stated clearly in the figure legends and Methods section. The relevance of the 2008 paper from the Efstathiou lab isn't obvious to us as it concerns viral gene expression during the establishment of latency in in a murine-infection model and does directly speak to what happens during reactivation in response to defined stimulus.

Referee #3:

In this manuscript from Hu et al, the authors use a combination of different single cell techniques to identify both a positive and negative regulator of Herpes Simplex Virus 1 reactivation. This is a well-executed study that will have a major impact on the field. The authors focus mainly on the negative regulator, Gadd45b and show that it is induced in neurons infected with HSV in response to a stimulus of reactivation (LY294002). Importantly, the induction was only detected in infected neurons and correlated with reduced UL36 gene expression (encodes a late viral protein) and reduced Us11-GFP positive neurons. Furthermore, expression of Gadd45b and Gadd45g inhibited HSV reactivation whereas Gadd45b knockdown resulted in spontaneous reactivation. This is a novel observation and as yet no role for Gadd45b/g in HSV reactivation has been identified. In addition, identification of a negative regulator that acts downstream of the initiation of gene expression is important as it highlights the potential for abortive reactivation. The authors make a number of additional observations that are important for the field including a thorough characterization at the single cell level of viral lytic mRNAs and the potential for expression from one or multiple viral genomes. The one weakness in the paper is the role for ICP4. The conclusion that Gadd45b suppresses viral late gene expression through the inhibition of the viral transcription factor, ICP4, is not well supported by the data.

We appreciate the Reviewer's positive comments about the manuscript regarding novelty and impact on the field.

1. *The data on ICP4 is correlative. The observations are 1) that a lack of detectible ICP4 correlates with Gadd45b puncta (but not nuclear staining) 2) that ICP4 mRNA levels are reduced at 72h post-LY with Gadd54b expression. However, expression of other mRNAs including another IE*

gene ICP27 and early gene UL30 are also reduced with Gadd45 expression, as is viral DNA load. Given that the authors show that as reactivation proceeds (and is more successful) the levels of viral mRNAs/cell increase, is it therefore possible that Gadd45b suppresses this general increase across the genome? It is surprising that the authors did not extend their smFISH assays from figure 2 to examine the consequence of Gadd45b expression on changes in viral mRNA levels over time and this would help strengthen the paper and address the potential for Gadd45b to prevent the transition to productive reactivation.

While we agree with the Reviewer's comment that the data on ICP4 is correlative, it is our view that the inhibitory effect on ICP4 protein levels (either direct or indirect) by Gadd45b ectopic expression in neurons provides the most plausible explanation of the phenotype we observed. Given the major role of ICP4 in regulating all classes of viral genes mostly positively (E,L) but also some negatively (IE genes), it's not hard to imagine some impact of Gadd45b on every marker, but the point we are trying to get across is that the true L genes are the most dependent on ICP4 (requiring both ICP4-dependent replication and transactivation) and thus are expected to be the most sensitive to reduced ICP4 levels. That being said, in light of the recommendations from Reviewers 1 and 3, we have now dramatically toned down this interpretation in the abstract and text.

2. A continued increase in viral mRNA between 48 and 72 hours could be indicative of cell spread. The authors should show that the stock of WAY150138 used is active in preventing cell-to-cell spread.

Agreed, this is an important control. Accordingly, we now provide evidence that our stock of WAY150138 inhibitor is active in preventing cell-to-cell spread during HSV-1 lytic infection of neurons (see **new Figure EV1C**). The images show that adjacent cells do not become infected upon treatment with WAY, confirming that the inhibitor prevents spread.

3. The authors interpret the data in Figure 2 as viral DNA replication dependent proteins are only made after 48 hours based on the detection of Us11-GFP compared to smFISH for ICP27, UL30 and UL36. The authors do not have enough data to make this conclusion. First, the smFISH assay is likely more sensitive than immunofluorescence and this may represent a limit of detection of viral protein. They also do not look at Us11 mRNA nor ICP27, UL30 or UL36 protein levels. It is also possible that GFP-detection is less sensitive. This is an important consideration in the HSV field and therefore the caveats need more thoroughly addressing. Immunofluorescence for additional viral proteins would strengthen the manuscript.

We agree with the Reviewer that differences in the timing at which protein products *and* mRNAs can be detected needs to be considered. In other words, that there are detection sensitivity differences between smFISH and immunofluorescence assays. We now provide new data that Us11 mRNA was similarly detected at early time-points (LY18 hrs) using RT-qPCR as another late gene UL36, with both peaking at LY72 hrs (**new Figure EV1D**). In contrast, Us11-GFP protein product did not appear until LY48 hrs.

4. A main conclusion of the paper on the role for Gadd45b inhibiting reactivation uses lentivirus mediated delivery of a transgene. The additional of lentivirus alone could have an effect on the neuronal cultures (the authors even see some spontaneous reactivation). The neurons also need to be transduced with a negative control lentivirus.

Again, this is an important point and we now provide new data including an empty version of the lentiviral vector as a negative control. This is a more rigorous control than mock infection, when

compared to lentiviral Gadd45b-MycFlag gene transduction and shows that lentivirus infection alone has only a subtle effect on the background level of reactivation in neuronal cultures and absolutely no inhibitory effect on LY-induced (or Mirin-induced) HSV-1 reactivation (**new Figure 6D and Figure EV4C**).

5. The EdU experiments permit the identification or viral DNA replication and are a great addition to the manuscript. However, EdU can have toxic effects. Therefore, the authors should show that the addition of EdU does not alter the subcellular localization of Gadd45b.

We now provide data showing that EdU treatment alone does not alter the subcellular localization of Gadd45b in uninfected, cultured neurons (**new Figure EV2D**). Importantly, EdU treatment alone did not lead to the presence or elevation of Gadd45b nuclear puncta staining (~0%), nor did it affect LY-induced Gadd45b pan-nuclear staining.

Minor points:

1. Multiple figures throughout the manuscript lack statistics. It would also be beneficial if the authors plotted individual biological replicates.

Noted, biological replicates and statistics have now been included in all of the data figures.

2. The authors do not mention in the methods how long EdU pulsing was carried out.

This was mentioned in the Methods section. The cells were pulse-labeled for 6 hrs at 37°C prior to fixation (page 21).

3. In figure 2D it would be clearer if the y-axis were labelled as % positive neurons that are double positive.

Noted.

4. It is not clear why Figure S3C is ICP27 mRNA were as the other panels show GFP-positive neurons.

We measured ICP27 mRNA induction because this in our hands this is one of the earliest measure of HSV-1 reactivation (see Hu et al., *Mol Cell* 2019; Kobayashi et al., *Genes Dev* 2012; Linderman et al. 2017 Cell Reports). Both Us11-GFP-positive detection or ICP27 mRNA induction are established methods in assessing HSV-1 reactivation from our published work as well as others (see Cliffe et al., *Cell Host & Microbe* 2015; Cuddy et al., *eLife* 2020).

Dear Dr. Huang,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees support the publication of your study. Referee #3 has a suggestion to improve the manuscript I ask you to address in a final revised version of the manuscript.

Moreover, I have these editorial requests:

- Please shorten the title to not more than 100 characters (including spaces).

- Please provide the abstract written in present tense throughout.

- We can accommodate only 5 EV figures. Please fuse some of the present EV figures to have not more than 5 EV figures in the final version. If a figure has only has 1 panel (like presently Fig EV5), we do not need the 'A'. Finally, please update all callouts accordingly in the manuscript text.

- There are call-outs to EV Tables 1 and 2, but I can't see these uploaded. Please check. In case these are missing, please upload the tables as separate files and add legends for these after the EV figure legends. In case these refer to Appendix tables, please update these callouts.

- Appendix Table S1 is a dataset. Please upload this file as Dataset EV1, change its name and its callout and add a legend on the first TAB of the excel file. Then please update the numbering of the Appendix tables and their callouts.

- Please upload the information in Appendix Table S5 as 'Reagents and Tools table' and remove the table from the Appendix. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. Please add the Appendix references to the main references. This example shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other": https://www.embopress.org/pb%2Dassets/embo-site/msb_177951_sample_FINAL.pdf

- Finally, please name the Appendix file just 'Appendix' and add page numbers (also to the TOC). Please add a title with name ('Appendix Table S1 - ...') above each table and a brief legend below the table. Please move the two Appendix files (computer codes) into the Appendix and name these Appendix Computer Code 1 and 2, and update their callouts in the manuscript text.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the diagrams in the Appendix), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment' or 'independent ereplicate', but clearly state if these were biological or technical replicates. If statistical testing was done but there is no significant difference, please also mark this in the diagrams (n.s.).

- Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently some scale bars are too thin and will not display well online (see e.g. those in panel 4H). Some panels have scale bars of different thickness (e.g. panel 1D).

- Please remove the paragraph 'Supporting information' from the manuscript text.

- Please correct 'Authors contributions' to 'Author contributions'.

- As the few Western blots shown are significantly cropped, could you please provide the source data for all the blots (main and EV figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number and send one PDF file per figure.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:

- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Best,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have sufficiently addressed the reviewers' comments.

Referee #2:

The authors have dealt adequately with this reviewer's concerns

---
Referee #3:

The authors have addressed all comments, including carrying out additional important controls and have amended the text so as to tone down the potential link to ICP4. I just have one very minor comment (see below). This is an incredibly interesting piece of work with numerous observations that will be important to the field.

Minor comment
The in vitro model system using Rat SCGs was described by the Wilcox and Johnson, 1987. This should be acknowledged on page 5 lines 4-5.

The authors have addressed all minor editorial requests.

Dr. Tony Huang
New York University School of Medicine
Biochemistry and Molecular Pharmacology
450 East 29th Street
Rm 807
New York, New York 10016
United States

Dear Dr. Huang,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case." Please note that the author checklist will still be published even if you opt out of the transparent process.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

****

THINGS TO DO NOW:

You will receive proofs by e-mail approximately 2-3 weeks after all relevant files have been sent to our Production Office; you should return your corrections within 2 days of receiving the proofs.

Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2021-53543V3 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Tony Huang
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2021-53543

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 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.

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.

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	in vitro studies cannot be blinded
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes, in Appendix Table S5
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Yes, All sequencing datasets generated as part of this study are available via the European Nucleotide Archive under the project accession PRJEB39022
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Yes
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A