EMBO reports

# HIV-1 Vpu induces neurotoxicity by promoting Caspase 3-dependent cleavage of TDP-43

Jiaxin Yang, Yan Li, Huili Li, Haichen Zhang, Haoran Guo, Xiangyu Zheng, Xiaofang Yu, and Wei Wei

*Corresponding author: Wei Wei (wwei6@jlu.edu.cn)*

## Review Timeline:

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| Submission Date: | 5th Mar 24 |
| Editorial Decision: | 15th Apr 24 |
| Revision Received: | 17th Jul 24 |
| Editorial Decision: | 8th Aug 24 |
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*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 Prof. Wei,

Thank you for the submission of your manuscript to EMBO reports. I 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, all referees 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 the concerns need to be addressed, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that the concerns of the referees must be addressed in the revised manuscript or in a 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. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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:

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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 labelled 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.

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Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
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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 in 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. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

# 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 now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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 labelled 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 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 for EV figures and all those in an Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

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

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11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions and do not provide your final manuscript text file with an author contributions section. See also our guide to authors:
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Materials and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software, and relevant equipment and including their sources and relevant identifiers), uploaded as separate file, followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points, to facilitate the adoption of the methodologies across labs. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in our author guidelines (section 'Structured Methods'):

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Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests 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
Senior Editor
EMBO Reports

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Referee #1:

In this article, Yang and colleagues demonstrated that HIV-1 infection induces neurotoxicity by the action of the HIV-1 accessory protein Vpu, which activated Caspase-3. They further showed that Vpu ectopic expression resulted in TDP43 cleavage, and inhibition of Caspase-3 attenuated HIV-1 mediated neurotoxicity.

Overall, the novelty of this work is limited. TDP43 is known to be cleaved by caspases and this work marginally increases our knowledge of viral-induced neurotoxicity or TDP-43 mediated neurotoxicity.

Major comments:
Figure 1. The protein ladder should be included in the western blots. what is the size of the TDP43 CTFs?

Figure 2I. Many times in dividing cells, TDP43 is transiently localized to the cytoplasm. This is the case for the cells shown here. This reviewer is not convinced that the Vpu+ panels where all TDP43 is mislocalized shows a correct representation of the entire well. See also Figure 2J where only 50% of cells has cytoplasmic TDP43. Please replace with a more representative set of images.

3. Are there accumulation of cryptic exons with the loss of nuclear TDP43?

4. The TDP43 antibody used in this study is not ideal because the immunogen is unknown. It is recommended that the authors use a number of different TDP43 antibodies widely used in the field:
- TDP43 C-terminal antibody (Proteintech 12892-1-AP)
- Total TDP43 antibody (Proteintech 10782-2-AP)
- Phospho-TDP43 (22309-1-AP)

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Referee #2:

The manuscript shows that the HIV-1 Vpu protein results in cleavage of TDP-43, translocation into the nucleus and aggregation, providing a further explanation for HIV-1-induced neurological damage.

While several published works, including those by this group, have shown viral proteases to cause the formation of TDP-43 aggregation, this has not been investigated for HIV. Furthermore, in this case, cleavage of TDP-43/ mis-localization and aggregation would seem to be due to Vpu activation of Caspase 3.

The conclusions drawn from the experimental data are reasonable.

Two points merit further investigation.

1. An aspect of TDP-43 proteinopathy not fully investigated is its loss of function. Is the amount of soluble TDP-43 enough to fulfil cellular functions?
For example; the mislocalization of TDP-43 in cells overexpressing Vpu is extensive, while for example in Figure 1 in the HIV-infected astrocyte cells that would express the Vpu protein at a more physiological level, this is less dramatic. Notwithstanding this, the change in the ratio of soluble to insoluble TDP-43 is substantial and more than would have been expected. It may be the case that nuclear aggregation of TDP-43 is also occurring. To test this the authors could also perform the solubility experiments on nuclear and cytoplasmic fractions. More importantly, however, the authors should investigate if there is a loss of TDP 43 function in cell lines infected with HIV, for example through analysis of known splicing targets of TDP-43. This would add a further layer of information regarding the effect of Vpu caspase activation and would significantly reinforce the manuscript's conclusions. In other words, the authors should investigate if, in the presence of the toxicity associated with the cleaved fragments and the mis-localization, there is also a loss of TDP-43 function in an HIV infection.

2. Can any evidence that cytoplasmic accumulation follows cleavage be provided? For example: HIV-1ΔVpu infection, does this result in a difference in cytoplasmic/nuclear TDP-43? Can the caspase inhibitors used in the HIV-infected cells and microscopy be performed or fractionation of the nuclear and cytoplasmic fractions be performed to evaluate the state of TDP-43 localization aggregation etc in the absence of the cleaved fragments but in an HIV infection background?

Minor corrections:
1. line 56-58- the authors should clarify that TDP-43 is not a pathological protein per se but its aggregation, reduced functionality, aberrant expression, mis-localization etc are associated with various neurological disorders. As the sentences stands this is not clear.
2. Caspase 3 activation has been seen to occur with several other components of HIV (i.e envelope proteins). In fact, Vpr whose overexpression did not result in cleaved TDP-43 fragments, has also been shown to activate Caspase 3. These aspects should be discussed.
3. No statistical significance is shown in the bar charts of 3F.
4. Was a comparison between the Vpu levels from the expression plasmid and from HIV infections made?

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Referee #3:

TAR DNA-binding protein (TDP-43). is a cellular protein with crucial roles in RNA splicing, transcription, mRNA transport, and mRNA stabilization. Here, the authors find that HIV-1 infection can induce cleavage and aberrant aggregation of TDP-43. The authors find that the aggregation and cleavage is caused by caspase 3, which is activated by the Vpu protein. The authors identify residue D89 in the 414 amino acid TDP-43 protein as the caspase 3 cleavage site. A second potential caspase 3 motif is apparently not used. Cleaved TDP-43 is cytotoxic in neural cells and expression of the cleaved TDP-43 CTF has dominant negative properties. Altogether, the study identifies TDP-43 as a novel target of Vpu and expression of Vpu results in the caspase-3 mediated cleavage od TDP-43.

The data presented here are interesting and make a reasonable case that Vpu is involved in the caspase-3-mediated cleavage of TDP-43. However, caspase-3 does not selectively target TDP-43 but cleaves a number of other cellular proteins as part of its involvement in host cell apoptosis. Caspase-3 is an effector caspase whose activation requires cleavage by an initiator caspase (e.g. caspase-9), which itself is activated through a pro-apoptotic signaling cascade. The authors are obviously not aware of a 2001 study (Akari et al; PMID: 11696595) which reports that Vpu induces apoptosis by suppressing the nuclear factor kappa B-dependent expression of antiapoptotic factors. Akari et al demonstrate that Vpu does not directly activate caspase-3. Instead, Vpu inhibits the expression of anti-apoptotic genes which normally prevent activation of caspase-3. Vpu does this by interfering with the activation of NFkB. Akari et al did not identify or characterize specific targets of caspase-3. Thus, the current study is a logical extension of the prior work and should be discussed in the context of the 2021 study.

If cleavage of TDP-43 by caspase-3 is unrelated to the general induction of apoptosis by Vpu, then treatment of cells with TNFalpha should result in Vpu independent cleavage of TDP-43. This should be tested.

What are the cytoplasmic aggregates shown in HIV-1 infected astrocytes in Fig. 1. The authors do not reveal what proteins are recognized by their HIV-1 specific antibody. However, there does not appear to be any colocalization. Research done with Vif and APOBEC3G previously revealed the presence of high molecular weight RNA protein complexes that include Vif and A3G. The authors should try to stain with specific antibodies to see if these aggregates include Vpu.

Fig. 2I suggests that Vpu expression induced TDP-43 translocation into the cytoplasm. How come, we do not see cytoplasmic aggregates as in Fig. 1A?

The authors state in their introduction that TDP-43 under normal conditions is predominantly localized to the nucleus. I therefore

assume that in the Ctrl samples in Fig 1C, TDP-43 is largely nuclear; yet , the fractionation into RIPA soluble and insoluble fraction shows TDP-43 in both fractions. Furthermore, histone is a nuclear marker and accumulates in the insoluble fraction. Based on that, TDP-43 would be read as mostly cytoplasmic in the ctrl sample since it is more prominent in the soluble fraction. This is highly confusing. I think the authors need to provide a detailed description of their fractionation analysis. In particular, they need to explain the difference between their RIPA/Urea fractionation and commercial fractionation kits that differentiate cytoplasmic, membrane, nuclear, and post-nuclear fractions.

A major technical weakness of the study is that methodologies are described in a very superficial manner. For instance, a key assay of this study is the "widely used" (line 88) cell fractionation assay that employs a combination of RIPA buffer and 7M urea extraction. Yet, there is no information on the buffer composition or the details of the sonication procedure.

Protein gels are sliced ultrathin and in most instances bands for full length TDP-43 and the CTF fragment are cut apart even though they can and should be shown on the same gel.

Molecular weight standards are missing throughout.

Figure 1: p55gag is RIPA buffer insoluble. Is that really true? What about p24 capsid?

Fig 3C: Vpu reduces levels of TDP-43-HA as expected but Z-DEVD-FMK treatment does not restore levels. Why not?

Fig 4B: labels in the cartoon are difficult to read. Also, the only obvious common feature in the two caspase-3 recognition motifs is the aspartic acid residue that appears to be the cleavage site. How were these motifs defined (provide a reference?)? Panel A suggests the presence of a 2nd aspartic acid residue upstream of the proposed cleavage site; however, the cartoon stops short of that (only shows VMDVFI).

Achim Breiling
Senior Editor
*EMBO Reports*

Dear Dr. Breiling,
Thank you very much for your letter of Apirl 15 concerning our manuscript entitled "**HIV-1 Initiates Neurotoxicity Via the Vpu/Caspase 3/TDP-43 Axis**"(EMBOR-2024-59135V1). We addressed each of the reviewers' points in the letter below and made relevant changes in the manuscript.

We are very grateful for the high quality reviews. It is obvious that each reviewer read the manuscript very carefully. We truly appreciate the feedback, which will substantially improve the reproducibility, quality and clarity of our paper.

Reviewers' comments:
Referee #1:
In this article, Yang and colleagues demonstrated that HIV-1 infection induces neurotoxicity by the action of the HIV-1 accessory protein Vpu, which activated Caspase-3. They further showed that Vpu ectopic expression resulted in TDP43 cleavage, and inhibition of Caspase-3 attenuated HIV-1 mediated neurotoxicity.
Overall, the novelty of this work is limited. TDP43 is known to be cleaved by caspases and this work marginally increases our knowledge of viral-induced neurotoxicity or TDP-43 mediated neurotoxicity.
**Response:** We thank the reviewer for the careful reading and constructive criticism. We have taken these points into serious consideration and revised the manuscript accordingly. Specific comments are also addressed below.

Major comments:
Figure 1. The protein ladder should be included in the western blots. what is the size of the TDP43 CTFs?
**Response:** As suggested by the reviewer, we have incorporated the protein ladder information into the revised figures (new Figures 1C, 1E, 1G, 1F-I, 2A-2H, 3A-3E, 4C-D, 5F, 5H). The TDP-43 C-terminal fragment (CTF) generated by HIV-1 Vpu has an approximate molecular weight of 35 kDa (page 5, lines 98-99).

Figure 2I. Many times in dividing cells, TDP43 is transiently localized to the cytoplasm. This is the case for the cells shown here. This reviewer is not convinced that the Vpu+ panels where all TDP43 is mislocalized shows a correct representation of the entire well. See also Figure 2J where only 50% of cells has cytoplasmic TDP43. Please replace with a more representative set of images.
**Response:** Thank you very much for this valuable suggestion. We have replaced the figures with images at a lower magnification as recommended (new Figure 2I).

3. Are there accumulation of cryptic exons with the loss of nuclear TDP43?
**Response:** We sincerely appreciate the reviewer for pointing this out. We have conducted the recommended experiments and demonstrated that HIV-1-induced cytoplasmic aggregation of TDP-43 results in the accumulation of cryptic exons of UNC13A, ATG4B and GSPM2. The data have been incorporated as new Appendix Figure S6 in the revised manuscript (page 11, lines 300-308).

4. The TDP43 antibody used in this study is not ideal because the immunogen is unknown. It is recommended that the authors use a number of different TDP43 antibodies widely used in the field:
- TDP43 C-terminal antibody (Proteintech 12892-1-AP)
- Total TDP43 antibody (Proteintech 10782-2-AP)
- Phospho-TDP43 (22309-1-AP)

**Response:** The provided suggestion is very helpful. We have successfully validated the HIV-1-induced cleavage and cytoplasmic aggregation of TDP-43 using the recommended antibodies (Total TDP43 antibody, Proteintech, 10782-2-AP and TDP43 C-terminal antibody, Proteintech 12892-1-AP), and we found that these antibodies are indeed more sensitive than those we used previously. The new data have been incorporated into the new Figure EV1 (page 5, lines 105-107).

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Referee #2:

The manuscript shows that the HIV-1 Vpu protein results in cleavage of TDP-43, translocation into the nucleus and aggregation, providing a further explanation for HIV-1-induced neurological damage.

While several published works, including those by this group, have shown viral proteases to cause the formation of TDP-43 aggregation, this has not been investigated for HIV. Furthermore, in this case, cleavage of TDP-43/ mis-localization and aggregation would seem to be due to Vpu activation of Caspase 3.

The conclusions drawn from the experimental data are reasonable.

**Response:** We greatly appreciate your constructive suggestions and the recognition of the potential importance of our work. We have taken these points into consideration and revised the manuscript accordingly. Specific comments are addressed below.

Two points merit further investigation.

1. An aspect of TDP-43 proteinopathy not fully investigated is its loss of function. Is the amount of soluble TDP-43 enough to fulfil cellular functions?

For example; the mislocalization of TDP-43 in cells overexpressing Vpu is extensive, while for example in Figure 1 in the HIV-infected astrocyte cells that would express the Vpu protein at a more physiological level, this is less dramatic.

**Response:** Thank you very much for pointing this out. We have measured the expression levels of Vpu in both Vpu overexpressed cells and HIV-1-infected cells. The immunoblotting data indicated that the levels of Vpu proteins were much lower in the latter group (refer to new Figure EV3B, page 6, lines 137-138).

Notwithstanding this, the change in the ratio of soluble to insoluble TDP-43 is substantial and more than would have been expected. It may be the case that nuclear aggregation of TDP-43 is also occurring. To test this the authors could also perform the solubility experiments on nuclear and cytoplasmic fractions.

**Response:** The reviewer is correct. As suggested by the reviewer, we have conducted the recommended experiments and demonstrated the aggregation of both cytoplasmic and nuclear TDP-43 proteins in the presence of HIV-1 infection. These data have been incorporated into our revised manuscript as Appendix Figure S2 (page 5, lines 114-119).

More importantly, however, the authors should investigate if there is a loss of TDP 43 function in cell lines infected with HIV, for example through analysis of known splicing targets of TDP-43. This would add a further layer of information regarding the effect of

Vpu caspase activation and would significantly reinforce the manuscript's conclusions. In other words, the authors should investigate if, in the presence of the toxicity associated with the cleaved fragments and the mis-localization, there is also a loss of TDP-43 function in an HIV infection.

**Response:** We sincerely appreciate this valuable suggestion. We have conducted the recommended experiments and demonstrated that HIV-1-induced TDP-43 dysfunction leads to an increasing accumulation of cryptic exons within UNC13A, ATG4B and GSPM2 (new Appendix Figure S6, page 11, lines 300-308).

2. Can any evidence that cytoplasmic accumulation follows cleavage be provided? For example: HIV-1ΔVpu infection, does this result in a difference in cytoplasmic/nuclear TDP-43? Can the caspase inhibitors used in the HIV-infected cells and microscopy be performed or fractionation of the nuclear and cytoplasmic fractions be performed to evaluate the state of TDP-43 localization aggregation etc in the absence of the cleaved fragments but in an HIV infection background?

**Response:** We thank the reviewer for this suggestion. We have conducted the recommended experiments and demonstrated that HIV-1ΔVpu infection or treatment with the caspase inhibitor z-VAD impairs the cytoplasmic accumulation of TDP-43 proteins triggered by HIV-1 (new Figures 3G and EV3A, page 7, lines 158-160 and page 6, lines 135-137).

Minor corrections:

1. line 56-58- the authors should clarify that TDP-43 is not a pathological protein per se but its aggregation, reduced functionality, aberrant expression, mis-localization etc are associated with various neurological
disorders. As the sentences stands this is not clear.

**Response:** We have corrected the description in the revised manuscript (page 3, lines 58-60).

2. Caspase 3 activation has been seen to occur with several other components of HIV (i.e envelope proteins). In fact, Vpr whose overexpression did not result in cleaved TDP-43 fragments, has also been shown to activate Caspase 3. These aspects should be discussed.

**Response:** As suggested by the reviewer, we have incorporated a relevant discussion in the revised manuscript (page 10, lines 273-278).

3. No statistical significance is shown in the bar charts of 3F.

**Response:** We have incorporated indicators of statistical significance in the revised Figure 3F.

4. Was a comparison between the Vpu levels from the expression plasmid and from HIV infections made?

**Response:** As suggested by the reviewer, we have conducted the recommended experiments and incorporated the immunoblotting data of Vpu expression as a new Figure EV3B (page 6, lines 137-138).

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Referee #3:

TAR DNA-binding protein (TDP-43). is a cellular protein with crucial roles in RNA splicing, transcription, mRNA transport, and mRNA stabilization. Here, the authors find

that HIV-1 infection can induce cleavage and aberrant aggregation of TDP-43. The authors find that the aggregation and cleavage is caused by caspase 3, which is activated by the Vpu protein. The authors identify residue D89 in the 414 amino acid TDP-43 protein as the caspase 3 cleavage site. A second potential caspase 3 motif is apparently not used. Cleaved TDP-43 is cytotoxic in neural cells and expression of the cleaved TDP-43 CTF has dominant negative properties. Altogether, the study identifies TDP-43 as a novel target of Vpu and expression of Vpu results in the caspase-3 mediated cleavage od TDP-43.

**Response:** We greatly appreciate the positive support from the reviewer and find the comments both insightful and helpful. Below are our point-by-point responses to the reviewer's critiques.

The data presented here are interesting and make a reasonable case that Vpu is involved in the caspase-3-mediated cleavage of TDP-43. However, caspase-3 does not selectively target TDP-43 but cleaves a number of other cellular proteins as part of its involvement in host cell apoptosis. Caspase-3 is an effector caspase whose activation requires cleavage by an initiator caspase (e.g. caspase-9), which itself is activated through a pro-apoptotic signaling cascade. The authors are obviously not aware of a 2001 study (Akari et al; PMID: 11696595) which reports that Vpu induces apoptosis by suppressing the nuclear factor kappa B-dependent expression of antiapoptotic factors. Akari et al demonstrate that Vpu does not directly activate caspase-3. Instead, Vpu inhibits the expression of anti-apoptotic genes which normally prevent activation of caspase-3. Vpu does this by interfering with the activation of NFkB. Akari et al did not identify or characterize specific targets of caspase-3. Thus, the current study is a logical extension of the prior work and should be discussed in the context of the 2021 study.

**Response:** We thank the reviewer for this suggestion. We have incorporated the relevant discussions into the revised manuscript (page 10, lines 270-273).

If cleavage of TDP-43 by caspase-3 is unrelated to the general induction of apoptosis by Vpu, then treatment of cells with TNFalpha should result in Vpu independent cleavage of TDP-43. This should be tested.

**Response:** The reviewer is correct. We have performed the suggested experiments and demonstrated that TNFalpha/cycloheximide (CHX) treatment can induce Vpu-independent TDP-43 cleavage and cytoplasmic aggregation (new Appendix Figure S4, page 7, lines 161-164).

What are the cytoplasmic aggregates shown in HIV-1 infected astrocytes in Fig. 1. The authors do not reveal what proteins are recognized by their HIV-1 specific antibody.However, there does not appear to be any colocalization. Research done with Vif and APOBEC3G previously revealed the presence of high molecular weight RNA protein complexes that include Vif and A3G. The authors should try to stain with specific antibodies to see if these aggregates include Vpu.

**Response:** --In Figure 1A, the green fluorescence represents the spatial distribution of GFP proteins expressed by the HIV-1 reporter viruses. We have improved the labeling in the revised Figure 1A to enhance clarity and precision.

**--**We thank the reviewer very much for this suggestion. We have conducted the recommended experiments and determined that the Vpu protein did not colocalize with TDP-43 aggregates, as observed by an immunofluorescence assay (new Appendix Figure S3, page 6, lines 141-143).

Fig. 2I suggests that Vpu expression induced TDP-43 translocation into the cytoplasm. How come, we do not see cytoplasmic aggregates as in Fig. 1A?

**Response:** To address this issue, we have improved the quality of the immunofluorescent pictures in the revised Figure 2I.

The authors state in their introduction that TDP-43 under normal conditions is predominantly localized to the nucleus. I therefore assume that in the Ctrl samples in Fig 1C, TDP-43 is largely nuclear; yet , the fractionation into RIPA soluble and insoluble fraction shows TDP-43 in both fractions. Furthermore, histone is a nuclear marker and accumulates in the insoluble fraction. Based on that, TDP-43 would be read as mostly cytoplasmic in the ctrl sample since it is more prominent in the soluble fraction. This is highly confusing. I think the authors need to provide a detailed description of their fractionation analysis. In particular, they need to explain the difference between their RIPA/Urea fractionation and commercial fractionation kits that differentiate cytoplasmic, membrane, nuclear, and post-nuclear fractions.

**Response:** We thank the reviewer for pointing this out. In this study, we used the RIPA/Urea fractionation assay, a commonly used method in the TDP-43 research field, to distinguish between RIPA-soluble and RIPA-insoluble proteins within various cellular contexts (Lg, Da Silva, et al. PMID:35112738, Winton, Matthew J. et al. PMID:18305110, Neumann, Manuela, et al. PMID:17023659). Histone H3 was employed as a marker to delineate the insoluble fraction (Audas,et al. PMID: 27720612). In contrast to conventional subcellular fractionation methods that use relatively gentle lysis approaches that preserve nuclear membrane integrity while disrupting cell membrane integrity, the RIPA/Urea fractionation assay combines RIPA lysis and sonication for complete disruption of both cell and nuclear membranes during lysis, followed by centrifugation to isolate insoluble protein components as precipitates (pages 4-5, lines 92-94).

A major technical weakness of the study is that methodologies are described in a very superficial manner. For instance, a key assay of this study is the "widely used" (line 88) cell fractionation assay that employs a combination of RIPA buffer and 7M urea extraction. Yet, there is no information on the buffer composition or the details of the sonication procedure.

**Response:** As suggested by the reviewer, we have incorporated these specific details into the revised manuscript (pages 14-15, lines 399-400,401,404-405).

Protein gels are sliced ultrathin and in most instances bands for full length TDP-43 and the CTF fragment are cut apart even though they can and should be shown on the same gel.

**Response:** As suggested by the reviewer, we have replaced these figures with unedited pictures (new Figures 1E, 2A-2B, 3A-3C, and 4C-4D).

Molecular weight standards are missing throughout.

**Response:** We have incorporated the molecular weight standards into the revised figures.

Figure 1: p55gag is RIPA buffer insoluble. Is that really true? What about p24 capsid?

**Response:** We thank the reviewer very much for this comment. We conducted solubility analyses of p55gag and p24CA in different human cells, and observed that p24CA in all tested cells was enriched mainly in the soluble fractions. However, in some cells, such

as T98G and HEK293T cells, p55gag proteins were mostly insoluble, whereas in SH-SY5Y and primary astrocyte cells, p55gag proteins were much more soluble (new Appendix Figure S1D-F). We noted that the differences in the solubility of p55gag in different cells did not influence the ability of HIV-1 infection to reduce TDP-43 solubility (page 5, lines 111-113).

Fig 3C: Vpu reduces levels of TDP-43-HA as expected but Z-DEVD-FMK treatment does not restore levels. Why not?

**Response:** We have consistently observed that the expression of Vpu can indiscriminately hinder ectopic protein expression but has no influence on endogenous protein expression, and this effect is not dependent on the activation of Caspase 3 by Vpu. We hypothesize that Vpu may exert an inhibitory effect on the functionality of elements within the expression vector, such as the promoter. To address this issue, we have included a relevant discussion in the revised manuscript (page 6, lines 153-156).

Fig 4B: labels in the cartoon are difficult to read.

**Response:** We have corrected this in the revised Figure 4B.

Also, the only obvious common feature in the two caspase-3 recognition motifs is the aspartic acid residue that appears to be the cleavage site. How were these motifs defined (provide a reference?)?

**Response:** We have added the related citations to the revised manuscript (page 7, lines 166-167).

Panel A suggests the presence of a 2nd aspartic acid residue upstream of the proposed cleavage site; however, the cartoon stops short of that (only shows VMDVFI).

**Response:** As suggested by the reviewer, we have corrected this issue in the revised Figure 4B.

Dear Prof. Wei

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from two of the three referees that I asked to re-evaluate the study, you will find below. Referee #1 declined to look into the revised manuscript but going through your p-b-p-response and the revised manuscript, I consider her/his points as adequately addressed. As you will see, the other two referees now fully support the publication of the study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- I would suggest this more comprehensive title:
HIV-1 Vpu induces neurotoxicity by promoting Caspase 3-dependent cleavage of TDP-43

- Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- 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 (main, EV and Appendix figures). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams. It seems that presently some diagrams have no or only partial stats or the 'n.s.' is missing. Moreover:
- Please note that the legends for figures EV 5b-c is not provided in the sequential manner (legend for figure 5c is provided before legend of figure 5b). This needs to be rectified.
- Please note that the exact p values are not provided in the legends of figures 1d; 2h; 3f; 4e; 5e, g, i; 6a, e.
- Please note that in figure EV 4b; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
- Please note that the white arrows are not defined in the legend of figure 1a. This needs to be rectified.

- Please add to each legend (main, EV and Appendix figures, where applicable) a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales. See:

https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please add scale bars of similar style and thickness to all microscopic images (also those in the Appendix), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, the scale bars are too thin and hard to see. Please improve.

- Please add a paragraph titled 'Biosafety' to the methods section gathering all information on where and how biosafety-relevant experiments with viruses were performed and that these were approved, and by whom (institution, government).

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, grants relate to the National Major Project for Infectious Disease Control and Prevention (2018ZX10731-101-001-016), the Department of Science and Technology of Jilin Province (No. 20210101015JC), the Open Project of Key Laboratory of Organ Regeneration and Transplantation, Ministry of Education, the Program for JLU Science and Technology Innovative Research Team (2017TD-08), and Fundamental Research Funds for the Central Universities are missing in the submission system. Please check.

- Please accept the tracked changes in the reagents and tools table.

In addition, I would need from you uploaded separately:
- 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 (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) 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
Senior Editor
EMBO Reports

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Referee #2:

The authors have responded to my questions and performed more tests as indicated. Alongside the work addressing the other reviewer's comments, I believe the manuscript has undergone considerable technical improvements and strengthened its conclusions.

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Referee #3:

The authors have adequately addressed my previous critiques. I am happy with the changes made to the manuscript

Achim Breiling
Senior Editor
*EMBO Reports*

Dear Dr. Breiling,
Thank you very much for your letter of August 8 concerning our manuscript entitled “HIV-1 Initiates Neurotoxicity Via the Vpu/Caspase 3/TDP-43 Axis” (EMBOR-2024-59135V2). We addressed each of the editor’s points in the letter below and made relevant changes in the manuscript.

We are very grateful for the high quality reviews. We truly appreciate the feedback, which will substantially improve the reproducibility, quality and clarity of our paper.

Editor's comments:
- I would suggest this more comprehensive title:
HIV-1 Vpu induces neurotoxicity by promoting Caspase 3-dependent cleavage of TDP-43
**Response:** We sincerely appreciate the editor for this suggestion. We have made the recommended change to the title.

- Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends
**Response:** We have confirmed the order of the manuscript sections as requested.

- 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 (main, EV and Appendix figures). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis
**Response:** As suggested by the editor, we have checked and confirmed that all the relevant information has been incorporated into the manuscript (page 21,

lines 647-648; page 22, lines 664-665 and 683-684; page 23, lines 696-697, lines716-717; page 24,lines 728-729; page 25, lines 779-780, lines 789-790).

If n<5, please show single datapoints for diagrams. It seems that presently some diagrams have no or only partial stats or the 'n.s.' is missing.
**Response:** We thank the editor for this suggestion, we have incorporated these items into the revised manuscript (new figures 2C-G, 4E, 5B, 5C, 5G, 6A, 6C-E, EV4B, EV4D, EV5D, S5, S6B. page21, lines 656-657; page23, lines 696, 703, 705, 713, 722, 726; page24, lines 727, 728; page 25, lines 770, 774-775, 789).

Moreover:
- Please note that the legends for figures EV 5b-c is not provided in the sequential manner (legend for figure 5c is provided before legend of figure 5b). This needs to be rectified.
**Response:** As suggested by the editor, the legends of Figure EV5 have been corrected as recommended (page 25, lines 784-786).

- Please note that the exact p values are not provided in the legends of figures 1d; 2h; 3f; 4e; 5e, g, i; 6a, e.
**Response:** The data analysis in this paper was performed using GraphPad commercial software. In this software, p-values are reported to four decimal places and rounded to "<0.0001" when they are less than 0.0001.

- Please note that in figure EV 4b; there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
**Response:** Thank you very much for poingting this out, we have corrected this item in the revised figure EV4b.

- Please note that the white arrows are not defined in the legend of figure 1a. This needs to be rectified.
**Response:** Thank you very much. We have included the description in the revised manuscript (page 21, lines 634).

- Please add to each legend (main, EV and Appendix figures, where applicable) a 'Data Information' section explaining the statistics used or providing information regarding replicates and scales. See:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat
**Response:** We have included a 'Data Information' section in each legend (page 21, lines 647-648; page 22, lines 664-665, and 683-684; page23, lines 696-697, lines 716-717; page 24, lines 728-729; page 25, lines 779-780 and 789-790).

- Please add scale bars of similar style and thickness to all microscopic images (also those in the Appendix), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, the scale bars are too thin and hard to see. Please improve.

**Response:** We have improved the scale bars in the revised figures 1A, 2I, 3G, 5A, 5D, 6B, 6F, EV1C, EV1D, EV3A, S3A, S4C, as recommended.

- Please add a paragraph titled 'Biosafety' to the methods section gathering all information on where and how biosafety-relevant experiments with viruses were performed and that these were approved, and by whom (institution, government).

**Response:** As suggested by the editor, we have incorporated these items into the revised manuscript (page 16, lines 436-439).

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Presently, grants relate to the National Major Project for Infectious Disease Control and Prevention (2018ZX10731-101-001-016), the Department of Science and Technology of Jilin Province (No. 20210101015JC), the Open Project of Key Laboratory of Organ Regeneration and Transplantation, Ministry of Education, the Program for JLU Science and Technology Innovative Research Team (2017TD-08), and Fundamental Research Funds for the Central Universities are missing in the submission system. Please check.

**Response:** Thank you very much for pointing this out. We have entered this information into the online submission system.

- Please accept the tracked changes in the reagents and tools table.

**Response:** We have corrected this item in the revised version.

In addition, I would need from you uploaded separately:
- 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 (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) 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.

**Response:** The required files have been uploaded separately as recommended by the editor.

Prof. Wei Wei
Jilin University
First Hospital
China

Dear Prof. Wei,

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.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: https://www.embopress.org/page/journal/14693178/authorguide#chargesguide

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

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Reports.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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>>> Please note that it is EMBO Reports policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/transparent-process#Review_Process

## EMBO Press Author Checklist

| | |
|---|---|
| Corresponding Author Name: Wei Wei | **USEFUL LINKS FOR COMPLETING THIS FORM** |
| Journal Submitted to: EMBO Reports | The EMBO Journal - Author Guidelines |
| Manuscript Number: EMBOR-2024-59135V1 | EMBO Reports - Author Guidelines |
| | Molecular Systems Biology - Author Guidelines |
| | EMBO Molecular Medicine - Author Guidelines |

**Reporting Checklist for Life Science Articles (updated January**
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

**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
- 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

**2. Captions**

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi^2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

**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 | N/A |

| 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:<br>- Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number<br>- Non-commercial: RRID or citation | Yes | Reagents and Tools Table |

| 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 | Reagents and Tools Table |

| 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 | Reagents and Tools Table |
| **Primary cultures:** Provide species, strain, sex of origin, genetic modification status. | Yes | Materials and Methods |
| Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination. | Yes | Materials and Methods |

| 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. | Not Applicable | N/A |
| **Animal observed in or captured from the field:** Provide species, sex, and age where possible**.** | Not Applicable | N/A |
| Please detail **housing and husbandry conditions**. | Not Applicable | N/A |

| 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 | N/A |
| **Microbes:** provide species and strain, unique accession number if available, and source. | Not Applicable | N/A |

| 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. | Not Applicable | N/A |

| 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? | Not Applicable | N/A |

**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 | N/A |
| Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable. | Not Applicable | N/A |

| 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 | N/A |

| 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 | Materials and 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? | Not Applicable | N/A |
| Include a statement about **blinding** even if no blinding was done. | Yes | Materials and Methods |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Not Applicable | N/A |
| 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 | Figure legends and Materials and Methods |

| 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 | Figure Legends |
| In the figure legends: define whether data describe **technical or biological replicates**. | Yes | Figure Legends |

## 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. | Not Applicable | N/A |
| 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. | Not Applicable | N/A |
| Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained. | Not Applicable | N/A |
| Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations. | Not Applicable | N/A |
| Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why. | Not Applicable | N/A |

| Dual Use Research of Concern (DURC) | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| 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 | N/A |
| If you used a select agent, is the security level of the lab appropriate and reported in the manuscript? | Not Applicable | N/A |
| If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript? | Not Applicable | N/A |

## 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.

| Adherence to community standards | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided. | Not Applicable | N/A |
| 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 | N/A |
| 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 | N/A |

## 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? | Not Applicable | N/A |
| 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 | N/A |
| Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Not Applicable | N/A |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Not Applicable | N/A |