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TDP-43 loss of function inhibits endosomal trafficking and alters trophic signaling in neurons

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Editor: Karin Dumstrei

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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

01 April 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see, the referees appreciate the manuscript, but also find that further analysis is needed to consider publication here. Referee #1 provides some very good and constructive comments and I would like to ask you to extend the analysis along those lines. Please also take into consideration the specific points raised by referee #2.

You can use the link below to upload the revised version. Please keep in mind that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

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

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

REFeree REPORTS

Referee #1:

This study looks at perturbed signaling pathways in response to TDP-43 loss. The RAB11-positive recycling endosome is the pathway under focus, and the authors document defects in the number of mobile recycling endosomes, but not in the stationary recycling endosomes. Through a proteomics approach, the authors identify VPS4B increase as a potentially key step in the pathway. Through a series of knock-down and over-expression studies, evidence is presented indicating that VPS4B increase is mediating the effect on endosomal trafficking. Further proteomics work then uncovers a potential role for reduced surface expression of the receptor ErbB4. These are interesting findings that may shed light on additional normal functions of nuclear TDP-43; however, certain aspects of the model are missing and the authors have not addressed the relationship of VSPB4 relative to the ErbB4 surface expression loss.

Issues that need to be addressed to improve the paper are as follows:

- 1) The authors show that TDP-43 knock-down promotes increased expression of VPS4B, and perform promoter-reporter assays to support this model. However, how is this occurring? Does TDP-43 bind to the VPSB4 promoter in the context of a properly chromatinized gene? ChIP experiments should be performed in cell lines and ideally on material from ALS patient iPSCs or their derivatives.
- 2) The authors identify reduced ErbB4 surface expression as an apparently independent event in TDP-43 nuclear loss-of-function cellular pathology. Have the authors looked for a connection between VPSB4 and ErbB4? If TDP-43 is not promoting ErbB4 expression at the RNA level, what is it doing?
- 3) The SALS patient analysis is an interesting result, but would it be possible to extend this analysis to TDP-43 ALS mutation carriers and C9orf72 ALS carriers to get a complete picture?
- 4) Is there increased VPS4B expression in frontal cortex of FTLT/ALS-TDP cases? I would ask that they extend the side-by-side comparison of FTLT/ALS-TDP and SALS cases. Fig 2F looks at β -2-transferrin levels in CSF of SALS cases and finds a statistically significant reduction. It would be good to test CSF of FTLT/ALS-TDP cases for this biomarker too. Secondly, it might be important (and the material would certainly be available) to test for VPS4B expression in SALS frontal cortex.

Minor issues for the authors to consider:

- Page 2 - references that TDP-43 inclusions are found in ALS & FTLT. This is very true but these inclusions may not be restricted to these disorders. TDP-43 inclusions can be linked with other neurodegenerative diseases such as Alzheimer's disease (AD). Please add references to this fact in the introduction, and comment on what it may mean to the conclusions for this study.
- It is supportive of the general thesis presented here that CHMP2B (an ESCRT-III protein) mutations are linked to ALS and FTLT, but they are extremely rare, and this should be pointed out.
- The citations are numbered in the results section (or at least in some sections of it), but the 'bibliography' is listed in an alphabetized form, please correct this. Particularly, ref's 18-19 as to why we should be interested in TMEM106B??
- There is a duplication of a few sentences on page 7.

Referee #2:

The manuscript by Schwenk and colleagues clearly demonstrates how TDP-43 loss of function affects recycling endosome trafficking and consequently the surface expression of receptors involved in dendrite growth.

This work opens new perspectives to uncover the effects of pathological TDP-43 in a previously unknown ALS-related pathway.

The authors have showed a strong relationship between TDP-43 and RAB11-positive endosomes. This relationship is mediated by the up-regulation of VPS4B occurring upon TDP43 knockdown.

The authors have used very interesting and novel approaches, and the results are consistent with the cellular mechanism proposed in the manuscript.

Since the topic is very specialized, in order to make the article easier to read, I would suggest to add some paragraphs in the introduction about the subject and explain in more detail the techniques used. There are few aspects that are taken for granted and that are only mentioned in the discussion, but by explaining them in the introduction and results sections would make the reading of the manuscript easier and more pleasant for the general reader.

Specific points:

- 1: The authors could show in Appendix Fig1 the kymographs regarding also the other GFP-tagged RAB proteins studied (RAB4, RAB5 and RAB7)
- 2: Please provide more defined IF panels for figure 2A, 6F and S6
- 3: Page. 5 line 14. The authors should briefly specify in the manuscript the RAB-11 dependent recycling pathway of transferrin.
- 4: Page. 7 line 12. There is a repeated paragraph in the text
- 5: Page. 8 line 2-5. The authors assert that a possible mechanism through which VPS4B could act in reducing recycling endosomal trafficking, may be explained by its function in promoting ESCRT-III disassembly. Could you explain more in detail the mechanism you think is behind the TDP knockdown/ VPS4B/ ESCRT-III axis?
- 6: Page. 8 line 17: The authors explain very well in the discussion that ErbB4 and FGFR1 proteins are chosen for further studies because of their involvement in dendrite growth. They could maybe add few lines also in the results paragraph in order to make the understanding of the chosen proteins easier.
- 7: Fig 7G-H: How do the authors explain a worst recovery in shCTR + ErbB4 transfected cells respect to shTDP + ErbB4? Do you think the presence of endogenous TDP-43 counteracts somehow the effect of the overexpression of ErbB4?
- 8: It would be interesting to show in live imaging, and respective kymograph, impaired movement and recycling of ErbB4. Have the authors already checked this aspect?

1st Revision - authors' response

30 June 2016

Point by point response to the reviewers

Referee #1:

This study looks at perturbed signaling pathways in response to TDP-43 loss. The RAB11-positive recycling endosome is the pathway under focus, and the authors document defects in the number of mobile recycling endosomes, but not in the stationary recycling endosomes. Through a proteomics approach, the authors identify VPS4B increase as a potentially key step in the pathway. Through a series of knock-down and over-expression studies, evidence is presented indicating that VPS4B increase is mediating the effect on endosomal trafficking. Further proteomics work then uncovers a potential role for reduced surface expression of the receptor ErbB4. These are interesting findings that may shed light on additional normal functions of nuclear TDP-43; however, certain aspects of the model are missing and the authors have not addressed the relationship of VSPB4 relative to the ErbB4 surface expression loss.

We thank the reviewer for his/her interest in our work and the constructive criticism. We addressed all point and strengthened the link between VSP4B and ErbB4 signaling further. Issues that need to be addressed to improve the paper are as follows:

- 1) The authors show that TDP-43 knock-down promotes increased expression of VPS4B, and

perform promoter-reporter assays to support this model. However, how is this occurring? Does TDP-43 bind to the VPSB4 promoter in the context of a properly chromatinized gene? ChIP experiments should be performed in cell lines and ideally on material from ALS patient iPSCs or their derivatives.

The reviewer raised a valid point. As suggested, we used ChIP to show interaction of TDP-43 with the promoter region of VPS4B in 293 cells, rat cortical neurons and human brain (new Figure 5C). Briefly, we analyzed a series of 8 amplicons in the 1kb promoter fragment used for the luciferase reporter assay and the corresponding region in the human promoter. For both the human and the rat sequence we could specifically amplify DNA from one GT-rich region in TDP-43 IPs in rat and human tissue. The sequence pattern in this region is consistent with previous data about TDP-43 affinity to DNA (Ou et al, J Virol 1995, Acharya et al., Dev Biol 2006). Together with the luciferase assay this data strongly suggests that endogenous TDP-43 negatively regulates VPS4B transcription in neurons.

2) The authors identify reduced ErbB4 surface expression as an apparently independent event in TDP-43 nuclear loss-of-function cellular pathology. Have the authors looked for a connection between VPSB4 and ErbB4? If TDP-43 is not promoting ErbB4 expression at the RNA level, what is it doing?

We present additional data to further strengthen our claim that loss of TDP-43 leads to reduced surface localization of ErbB4 by impaired trafficking of recycling endosomes, which is mediated by VPS4B upregulation.

- a) ErbB4 was identified through its reduced surface expression in TDP-43 knockdown neurons by proteomics done with the aim to find critical downstream effects of the inhibition recycling endosome trafficking (now Figure 7).
- b) TDP-43 knockdown delays surface delivery of HA/T-ErbB4 after all HA-epitopes were removed from the cell surface using thrombin cleavage confirming that TDP-43 knockdown inhibits membrane insertion of ErbB4 (new Fig. 8).
- c) VPS4B overexpression also reduces the surface expression of ErbB4 (new Figure EV5 A/B) suggesting that VPS4B upregulation drives the effects of TDP-43 knockdown on receptor trafficking.
- d) VPS4B overexpression prevents stimulation of dendrite growth by the ErbB4 ligand NRG1 suggesting that VPS4B drives the effects of TDP-43 on ErbB4 signaling (new Figure EV5 C/D).
- e) The blunted dendrite development in VPS4B overexpression neurons can be increased by ErbB4 overexpression (new. Figure EV5 E/F).

Together, these data strongly suggest that impaired trafficking due to VPS4B upregulation is causing the reduced ErbB4 surface expression in TDP-43 knockdown neurons. These findings are now clearly discussed in the revised manuscript.

3) The SALS patient analysis is an interesting result, but would it be possible to extend this analysis to TDP-43 ALS mutation carriers and C9orf72 ALS carriers to get a complete picture?

We extended our CSF analysis as requested. Since FTLN is a much more heterogenous disease than ALS, it is much more difficult to get high sample numbers for all requested subgroups. We now analyzed CSF from FTD-ALS and behavioral variant FTD patients, because they are most likely to have TDP-43 pathology (new Figure 2C). Unfortunately, CSF samples from true FTD-ALS are rare even in the large biobank at the Ulm university, because most patients only match the strict clinical criteria for diagnosis of FTD-ALS usually at the disease end stage and often decline additional CSF sampling at this point. We found reduced b2 transferrin levels in both groups, although this did not reach statistical significance. While conducting these experiments we also measured additional controls and ALS patients (all grouped together in the new Figure 2C). In this replication cohort b2 transferrin reduction in ALS cases was reproducible and statistically significant. The FTD-ALS group contains three C9orf72 mutation carriers, whose b2 transferrin values are similar to the other cases. CSF from TDP-43 mutation carriers was not available to us.

4) Is there increased VPS4B expression in frontal cortex of FTLN/ALS-TDP cases? I would ask that they extend the side-by-side comparison of FTLN/ALS-TDP and SALS cases. Fig 2F looks at β -2-transferrin levels in CSF of SALS cases and finds a statistically significant reduction. It would be good to test CSF of FTLN/ALS-TDP cases for this biomarker too. Secondly, it might be important (and the material would certainly be available) to test for VPS4B expression in SALS frontal cortex.

Parts of this data was already included in the original submission (Figure S6C-F) and was now shifted and extended to the main Figure 5. We replicated our findings of increased VPS4B levels in FTLN/ALS cases in the spinal cord (new Fig. 5F/G). As a control we now show that ALS patients lacking TDP-43 pathology in the frontal cortex show normal VPS4B levels as requested, while they have increased levels in the spinal cord (new Fig. EV4 A-D).

The comment regarding CSF analysis have already been addressed above (point 3).

Minor issues for the authors to consider:

- Page 2 - references that TDP-43 inclusions are found in ALS & FTLN. This is very true but these inclusions may not be restricted to these disorders. TDP-43 inclusions can be linked with other neurodegenerative diseases such as Alzheimer's disease (AD). Please add references to this fact in the introduction, and comment on what it may mean to the conclusions for this study.

It is true data our data may have implications for other diseases with TDP-43 pathology, most importantly Alzheimer's diseases. We added this information to the introduction and the discussion and provide key references.

- It is supportive of the general thesis presented here that CHMP2B (an ESCRT-III protein) mutations are linked to ALS and FTLN, but they are extremely rare, and this should be pointed out. We pointed that out in introduction and discussion as suggested.

- The citations are numbered in the results section (or at least in some sections of it), but the 'bibliography' is listed in an alphabetized form, please correct this. Particularly, ref's 18-19 as to why we should be interested in TMEM106B??

We fixed these formatting errors and apologize for the confusion.

- There is a duplication of a few sentences on page 7.

We removed the duplicated sentences.

Referee #2:

The manuscript by Schwenk and colleagues clearly demonstrates how TDP-43 loss of function affects recycling endosome trafficking and consequently the surface expression of receptors involved in dendrite growth.

This work opens new perspectives to uncover the effects of pathological TDP-43 in a previously unknown ALS-related pathway.

The authors have showed a strong relationship between TDP-43 and RAB11-positive endosomes.

This relationship is mediated by the up-regulation of VPS4B occurring upon TDP43 knockdown.

The authors have used very interesting and novel approaches, and the results are consistent with the cellular mechanism proposed in the manuscript.

Since the topic is very specialized, in order to make the article easier to read, I would suggest to add some paragraphs in the introduction about the subject and explain in more detail the techniques used. There are few aspects that are taken for granted and that are only mentioned in the discussion, but by explaining them in the introduction and results sections would make the reading of the manuscript easier and more pleasant for the general reader.

We appreciate the reviewer's interest and suggestions. We extended our introduction to cover TDP-43 function and the ESCRT complex more extensively and now explain the live imaging technique, the transferrin recycling assay and the Sholl analysis better in the results.

Specific points:

1: The authors could show in Appendix Fig1 the kymographs regarding also the other GFP-tagged RAB proteins studied (RAB4, RAB5 and RAB7)

These results had already shown in the supplemental data of the original submission. We now promoted these Figures to Expanded View format to make them more accessible (now Figures EV1 and 2).

2: Please provide more defined IF panels for figure 2A, 6F and S6

We agree that some of the panels did not look nice in printouts. We always show images from the same experiments that we quantified, but to allow correct quantification we had to avoid oversaturation of pixels and therefore the images are not that bright. We now inverted some of the images to increase the visibility of details in printouts and added the missing scale bars. If requested, we could also adjust brightness and contrast for optimal visibility.

3: Page. 5 line 14. The authors should briefly specify in the manuscript the RAB-11 dependent recycling pathway of transferrin.

We added a short paragraph in the main text to better explain RAB11 dependent transferrin recycling.

4: Page. 7 line 12. There is a repeated paragraph in the text

We removed this duplicated section and apologize for the confusion.

5: Page. 8 line 2-5. The authors assert that a possible mechanism through which VPS4B could act in reducing recycling endosomal trafficking, may be explained by its function in promoting ESCRT-III disassembly. Could you explain more in detail the mechanism you think is behind the TDP knockdown/ VPS4B/ ESCRT-III axis?

In post-mitotic neurons the ESCRT complex mainly regulates the flow of early endosomes into multivesicular bodies or recycling endosomes. VPS4B upregulation is thought to promote ESCRT disassembly. We now show that overexpressing ALIX/PDCD6IP, which promotes ESCRT assembly and thus antagonizes VPS4B, also rescues trafficking of recycling endosomes (new Appendix Figure S3). This further supports our claim that VPS4B upregulation is the main driver of impaired recycling endosome trafficking in TDP-43 knockdown neurons. We additionally enhanced the introduction and discussion to better cover the TDP-43/VSP4B/ESCRT axis.

6: Page. 8 line 17: The authors explain very well in the discussion that ErbB4 and FGFR1 proteins are chosen for further studies because of their involvement in dendrite growth. They could maybe add few lines also in the results paragraph in order to make the understanding of the chosen proteins easier.

We added a brief rationale for choosing ErbB4 and FGFR1 in the result section as suggested.

7: Fig 7G-H: How do the authors explain a worst recovery in shCTR + ErbB4 transfected cells respect to shTDP + ErbB4? Do you think the presence of endogenous TDP-43 counteracts somehow the effect of the overexpression of ErbB4?

At the first glance, the stronger effect of ErbB4 in TDP-43 knockdown neurons is indeed surprising. However, the effect of ErbB4 expression is comparable to the effect of NRG1 stimulation in controls (Fig. 9f vs. 9h). Thus, NRG1/ErbB4 signaling may be near saturation under control conditions. In contrast, TDP-43 knockdown presumably inhibits the signaling of several additional trophic receptors, explaining why stimulating the system with ErbB4 expression under these deprived conditions may have a bigger effect.

8: It would be interesting to show in live imaging, and respective kymograph, impaired movement and recycling of ErbB4. Have the authors already checked this aspect?

We attempted these experiments using an ErbB4-GFP construct. However, ErbB4-GFP expression levels sufficient for live imaging were too toxic to allow a meaningful experiment. Instead we used a thrombin cleavage assay to provide direct evidence for impaired ErbB4 recycling in TDP-43 knockdown neurons (new Figure 8). Briefly, we expressed HA/T-ErbB4 and removed all HA epitopes by thrombin cleavage in live cells. Recovery of HA/T-ErbB4 at the cell surface from internal stores was delayed in TDP-43 knockdown neurons further suggesting that TDP-43 loss of function inhibits trafficking of recycling endosomes.

2nd Editorial Decision

26 July 2016

Thanks for submitting your revised manuscript to The EMBO journal. Your revision has now been seen by the two referees and their comments are provided below.

As you can see both referees appreciate the introduced revisions and support publication here. There is a minor issue raised by referee #1 that should be sorted out. You can use the link below to upload the revised manuscript.

REFeree REPORTS

Referee #2:

The revised manuscript is substantively improved over the initial version, and all of my criticisms have been adequately addressed.

My only remaining minor concern is as follows: The ChIP data in Figure 5 is presented as PCR amplification products on gels, but should be quantified.

Referee #3:

The authors have met all the requests of this referee

2nd Revision - authors' response

11 August 2016

Point by point response:

Referee #2:

The revised manuscript is substantively improved over the initial version, and all of my criticisms have been adequately addressed.

We would like to thank the referee for his approval of our revised version.

My only remaining minor concern is as follows: The ChIP data in Figure 5 is presented as PCR amplification products on gels, but should be quantified.

We now quantified the gels from at least three independent experiments for each ChIP condition (cortical neurons, HEK293 cells and human brain) and added the result as a new Figure 5D.

Referee #3:

The authors have met all the requests of this referee

We would like to thank the referee for his approval of our revised version.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dieter Edbauer

Journal Submitted to: The EMBO Journal

Manuscript Number:

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 $n \leq 5$, 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 χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	Sample size was chosen according to experience and studies with comparable cell biological / biochemical experiments. A common number of 3-6 experiments was performed
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	no animal studies were conducted
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	no samples were excluded
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	no animal studies were conducted
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.	morphological assesment of neurons and image aquisition and quantification of brain slices was done blinded to the experimental conditions
4.b. For animal studies, include a statement about blinding even if no blinding was done	no animal studies were conducted
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, data met assumption od the tests. Data was tested for normal distribution with D'Agostino-Pearson omnibus normality test. If data points were not normally distributed, a non-parametric test was used, when applicable.
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>

<http://1degrebio.org>

<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<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-tun>

<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://ijl.biochem.sun.ac.za>

http://oba.od.nih.gov/biosecurity/biosecurity_documents.html

<http://www.selectagents.gov/>

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).	All the antibodies were purchased from commercial sources, catalog number is given in the method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK293FT cells were purchased by Life Technology and routinely tested for mycoplasma contamination.

* 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.	no animal studies were conducted
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	no animal studies were conducted
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.	no animal studies were conducted

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	no experiments with human subjects were conducted
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.	no experiments with human subjects were conducted
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	no experiments with human subjects were conducted
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	no experiments with human subjects were conducted
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	no experiments with human subjects were conducted
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.	no experiments with human subjects were conducted
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.	no experiments with human subjects were conducted

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	proteomic data sets are provided in Appendix Tables S1 and S2
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).	proteomic data sets are provided in Appendix Tables S1 and S2
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).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: Primary Data Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 Referenced Data Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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.	no computational models are used

G- Dual use research of concern

23. 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
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