EMBO reports

Role of PVN-sympathetic-adipose circuit in depression and insulin resistance under chronic stress

Jing Wang, Linshan Sun, Jingjing You, Honghai Peng, Haijing Yan, Jiangong Wang, Fengjiao Sun, Minghu Cui, Sanwang Wang, Zheng Zhang, Xueli Fan, Dunjiang Liu, Cuilan Liu, Changyun Qiu, Chao Chen, Zhicheng Xu, Jinbo Chen, Wei Li, and Bin Liu
DOI: 10.15252/embr.202357176

Corresponding author(s): Bin Liu (liubin3714@126.com), Wei Li (yishengliwei@163.com)

Review Timeline:

Submission Date:	13th Mar 23
Editorial Decision:	19th Apr 23
Revision Received:	2nd Aug 23
Editorial Decision:	14th Sep 23
Revision Received:	20th Sep 23
Editorial Decision:	29th Sep 23
Revision Received:	29th Sep 23
Accepted:	9th Oct 23

Editor: Esther Schnapp / Deniz Senyilmaz Tiebe

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Liu

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also raise several concerns that should be addressed.
Referee 1 points out that the conclusion that PVN activation connects depression and insulin resistance/autonomic changes in fat is not appropriate. Please make sure that all overinterpretations are removed from the manuscript. Also, the experimental methods need to be described in more detail and more thoroughly.
Both referees 1 and 3 mention that CRH and CORT levels should be analyzed. In principle, all referee concerns should be addressed, but please let me know in case you disagree, and we can discuss the revisions further, also in a video chat, if you wish. For example, not all points raised by referee 3 might have to be addressed.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (20th Jul 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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

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

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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

manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/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 & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. 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.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n Program fragment delivered error ``Can't locate object method "less" via package "than" (perhaps you forgot to load "than"?) at //ejpvfs23/sites23b/embor_www/letters/embor_decision_revise_and_review.txt line 56.' 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In the manuscript, "Role of PVN-sympathetic-adipose circuit in depression and insulin resistance under chronic stress" the authors describe experiments showing behavioral and physiological changes in response to chronic restraint stress and activation of neurons in the PVN. The experiments nicely show that activation of a population of neurons that are activated by stress in the PVN can cause changes in autonomic innervation of white adipose tissue. They also show that chronic restraint stress causes changes in depressive like behavior. However, the central premise of the paper is that there is a mechanistic connection between behavioral changes caused by stress, and physiological changes in the periphery, which is not addressed experimentally in the paper. Moreover, the results from gene expression and CHIP analysis in fat do not provide any mechanistic insight into the connection between behavioral and physiologic changes caused by chronic stress. While many of the experiments are very innovative and difficult, without demonstrating a connection between behavioral and physiologic changes, the primary assertion of the paper - that it connects depression and insulin resistance/autonomic changes in fat- is not an appropriate conclusion. Below I list the major and minor issues that led me to this conclusion:
Major issues:
1. The premise of the paper is that there is a PVN-sympathetic-adipose circuit that mediates both behavioral and physiologic responses to restraint stress. However, in many cases it seems that PVN activation can drive physiologic changes without altering behavior. This is the most evident in the dreadd activation experiments where PVN neurons were chronically activated, which resulted in physiologic changes in the fat as well as changes in glucose tolerance, but did not alter behavior. The authors do show that PVN neuron activation sensitizes animals to restraint induced changes in behavior, suggesting that behavioral changes only occur with activation of the complete repertoire of stress responses evoked by restraint. The other experiment that suggests behavioral and physiological changes are not causally linked is the beta antagonist experiment, in which behavioral changes were ameliorated but WAT innervation was unchanged. Together these results suggest that, while PVN activation is common to behavioral and physiologic changes, they most likely are parallel phenomena and may not be causally linked. If the authors were to provide experimental evidence that behavioral changes are due to changes in the periphery (which I believe is their assertion), I would be more supportive of the manuscript.
2. The PVN is well known as the source of stress-released CRF, which drives HPA axis activity leading to elevated levels of circulating corticosterone. It is likely that the neurons that are activated by stress, and that the authors manipulate using dreadds, are, in part, CRF neurons that drive elevations of Cort. Cort can have profound effects on transcription in target tissues, such as fat. The authors describe changes in gene expression in fat in response to chronic restraint stress but never discuss or test the relative importance of changes in Cort compared with sympathetic innervation. By not addressing Cort as a possible mediator of correlated behavioral and physiological influences of the PVN, the authors leave a gap in their study. The authors should provide some experiments that address how manipulation of PVN neurons with dreadds alters Cort levels, and potentially how cort modulates gene expression in WAT to address this omission.
Minor issues:
1. In all of the figures, pictures of the brains should be at higher magnification and include landmarks of the areas of interest. As presented, it is hard to see colocalization of the markers being tested.
2. For the pseudorabies tracing experiments, it is unclear how many synapses are transited by the virus before it reaches the PVN. The timing after infection is critical, and maybe the authors could show a tim ecourse that would indicate the number of synapses between the PVN and adipose tissue.
3. In the description of the chronic dreadd activation experiment, it is unclear when CNO was injected to chronically activate/inhibit PVN neurons. Was CNO given once per day for each of 42 days?
4. The manuscript should be edited for English usage. Although it is clearly written, there are many grammatical errors.

Referee #2:

In this manuscript, the Authors explore the role of PVN-sympathetic-adipose circuit in depression and insulin resistance under chronic stress. The Authors note that chronic restraint stress increases sympathetic innervation in white adipose tissue (WAT) and is associated with impaired glucose and insulin metabolism as well as depressive-like behaviors. The Authors also determine that restraint stress activates neurons in the PVN and that chemogenetic activation of the PVN is sufficient to upregulate sympathetic innervation in WAT. Given that sympathetic innervation activates β3-adrenergic receptor signaling in adipocytes, the Authors further show that pharmacological blockade of the β3-adrenergic receptor ameliorates depressive-like behaviors and insulin metabolism in a chronic restraint model. The Authors also characterize the expression of several adipokines in the chronic restraint and PVN activation models and further explore their transcriptional regulation.
The manuscript under review addresses a timely topic and represents a welcome addition to the literature linking chronic stress

with depression and metabolic disorders. The manuscript is, therefore, of potential interest to a broad readership, ranging from the fields of metabolism to neuroscience and physiology.
Given the interesting nature of the topic at hand, it is my opinion that the Authors could improve several aspects of this manuscript:
• In Figure 2B, the Authors show colocalization of neurons traced from the white adipose tissue (WAT) with neurons activated (cFos positive) by restraint stress. It would be important to show whether this experimental model induces any neuronal activation even without exposure to stress. PRV tracing plus cFos staining in control mice would be informative.
• In Figure 2D, the experimental details are not clear. The Authors should clarify whether the CNO injection was performed just one time or daily for 42 days. Along the same line, in Figure 2G and 2H the experimental details are not sufficiently explained. It is not clear whether the experimental mice were injected with the CNO during the additional 6 days of restraint stress or not. It is also not obvious to what procedures the control mice were subjected. Similarly, in Figure 2K and 2L, it is not clear whether the GTT and ITT was performed without the additional restraint stress regimen or nor. It is difficult to interpret the results without confirming the experimental details.
• L748337 is a β3-adrenergic receptor antagonist and this information could be added to the manuscript. In Figure 3, the Authors could explain better the experimental details. It is not clear whether the L748337 was administered daily and to what procedures the control mice were subjected.
• In the sub-chapter "Regulation of adipokines related to depression and insulin resistance under chronic restraint stress", the Authors conclude: "Taken together, the findings suggested that decreased levels of adipokines, including leptin, adiponectin, Angptl4 and Sfrp5, may account for the depressive-like behaviours and insulin resistance induced by chronic stress or chronic activation of PVN neurons". While this correlation seems to be correct for the observed WAT changes in chronic restraint stress model, it is not entirely accurate for the PVN activation. The adiponectin levels in WAT (both gene expression and protein) where increased in mice expressing activatory Dreadds in the PVN. The Authors should acknowledge and discuss these discrepancies.
• It is well established that activation of the sympathetic outputs in WAT drives lipolysis. It would be interesting to see whether the lipolytic pathway in adipocytes and the FFA levels in the blood are affected in the chronic restraint stress and PVN activation models. The Authors show convincing images of WAT sympathetic innervation, but what happens to WAT histology and the mass of WAT in both experimental models?

Other points on the accompanying material:
• In plots showing the RT-PCR data it is not clear how the relative gene expression was calculated.
• Figure legends should include information about the statistical analyses that were used.
• In Figure 1A, the timeline should be labeled with "days"
• There is a typo in Figure 2J ("locomptor")

The Authors are encouraged to correct some grammar/text issues; examples:
• "Chronically activation of PVN neurons responding..."
• "These ChIP results may could explain

Referee #3:

This manuscript uncovers a new circuity composed by PVH-SNS-adipose tissue, regulating insulin resistance and depression in a context of chronic restraint stress. It shows how these comorbidities have in common the adipose tissue as an endocrine organ involving the secretion of different adipokines, and how the innervation of this tissue by the SNS is also orchestrated by the PVH.

The authors performed elegant techniques, and experimental designs to show how the stress restraint induced morphological changes in the adipose tissue, as well as to elucidate the central regulation of this process. The manuscript is well written and relevant. Some comments need to be addressed.

1. Overall, the manuscript could better benefit from a more elaborated explanation on how this chronic stress restraints translate into "depression" or a "depressive" state of the mice. The authors wanted to emphasize the transnationality of their study to human depressive states and insulin resistant and I am not quite sure if the model used, as well as de behavioral test performed, are the most suitable ones. The authors can still point out on discussion the relation to depression and insulin resistance.
2. One limitation is that authors after the pseudorabies virus tracing experiment plus the cFos staining go directly to the PVH. I guess there should be also other brain regions receiving inputs from the SNS (such as DMH or VMH, as the authors pointed in the introduction), as well as other cFOS positive brain areas after the chronic restraint. Including more brain regions images with positive retrotracing or cFOS positive neurons, would increase the quality of the paper.
3. In accordance with the previous point, knowing other brains areas that are not involved in this restraint activation of neuronal populations (performing activating, or inhibiting neuronal experiments in these areas with the same viral approach than the PVH), would strengthen the conclusions.
4. For GTT and ITT analysis, showing the area under the curve or performing HOMA-IR analysis would reinforce results.

5. The PVH also receives inputs from the ARC, and it is also involved in the regulation of food intake. Could the authors also provide food intake data on their chronic restraint paradigm?
6. It is also mentioned through the manuscript the HPA axis might also have a role in regulating chronic stress and depression. Could the authors provide some data on how is the state of this axis on their models? Have authors assayed CRH mRNA and/or protein levels in the PVH? Circulating ACTH or CORT levels?
7. Do the authors have any guess on which neuronal population might be mediating the effects of chronic restraint within de PVH? In keeping with the former point, could CRH neurons be involved?
8. In the experimental paradigm of the β-antagonist, it is unclear the frequency of the treatment, please explain in the Methods or the Figure Legend. Also, please indicate in Figure 5, if the stress paradigm is the same than the previous one, It is not really clear in the text.
9. Could the authors provide some insight on why restraint induce a negative regulation of adiponectin (both at RNA and protein levels) but chronic activation of PVH neurons, induced the opposite effect?

Referee #1:

In the manuscript, "Role of PVN-sympathetic-adipose circuit in depression and insulin resistance under chronic stress" the authors describe experiments showing behavioral and physiological changes in response to chronic restraint stress and activation of neurons in the PVN. The experiments nicely show that activation of a population of neurons that are activated by stress in the PVN can cause changes in autonomic innervation of white adipose tissue. They also show that chronic restraint stress causes changes in depressive like behavior. However, the central premise of the paper is that there is a mechanistic connection between behavioral changes caused by stress, and physiological changes in the periphery, which is not addressed experimentally in the paper. Moreover, the results from gene expression and CHIP analysis in fat do not provide any mechanistic insight into the connection between behavioral and physiologic changes caused by chronic stress. While many of the experiments are very innovative and difficult, without demonstrating a connection between behavioral and physiologic changes, the primary assertion of the paper - that it connects depression and insulin resistance/autonomic changes in fat- is not an appropriate conclusion. Below I list the major and minor issues that led me to this conclusion:

Major issues:

1. The premise of the paper is that there is a PVN-sympathetic-adipose circuit that mediates both behavioral and physiologic responses to restraint stress.

However, in many cases it seems that PVN activation can drive physiologic changes without altering behavior. This is the most evident in the dreadd activation experiments where PVN neurons were chronically activated, which resulted in physiologic changes in the fat as well as changes in glucose tolerance, but did not alter behavior. The authors do show that PVN neuron activation sensitizes animals to restraint induced changes in behavior, suggesting that behavioral changes only occur with activation of the complete repertoire of stress responses evoked by restraint. The other experiment that suggests behavioral and physiological changes are not causally linked is the beta antagonist experiment, in which behavioral changes were ameliorated but WAT innervation was unchanged. Together these results suggest that, while PVN activation is common to behavioral and physiologic changes, they most likely are parallel phenomena and may not be causally linked. If the authors were to provide experimental evidence that behavioral changes are due to changes in the periphery (which I believe is their assertion), I would be more supportive of the manuscript.

We are deeply thankful for the critiques and suggestions. The paraventricular thalamic nucleus (PV) activated under restraint stress which wasn't labelled by pseudorabies virus (PRV) until the 6th day since PRV was injected into iWAT or eWAT (Fig EV4A-4F). Chronical activation of PV with the same viral approach than the PVN didn't affect the sympathetic innervation in WAT (Fig EV4G-4L), the depression level or insulin sensitivity (Fig EV4M-4R). These

results suggested a correlation between increased sympathetic innervation of adipose and depressive-like behaviours and insulin resistance, and highlighted the importance of PVN in the increased sympathetic innervation, depressive-like behaviours and insulin resistance under chronic restraint stress. To draw the conclusion that the increased sympathetic innervation and depressive-like behaviours are causally linked, further studies are needed.

2. The PVN is well e expression in fat in response to chronic restraint stress but never discuss or test the relative importance of changes in Cort compared with sympathetic innervation. By not addressing Cort as a possible mediator of correlated behavioral and physiological influences of the PVN, the authors leave a gap in their study. The authors should provide some experiments that address how manipulation of PVN neurons with dreadds alters Cort levels, and potentially how cort modulates gene expression in WAT to address this omission.

We are grateful to the reviewer by this valuable advice. As the HPA axis also has a role in regulating insulin resistance and depression, we assayed the CRH mRNA and protein in the PVN, the serum ACTH and CORT levels, under both chronic restraint stress and PVN activation paradigms (Fig EV5). Our results showed that chronic restraint treatment increased the serum CORT level (Fig EV5E), while chronical activation of PVN didn't affect the serum CORT level (Fig EV5J). Previous studies suggested that CORT inhibited the expression of adiponectin[1,2]. In our study, the serum CORT level increased,

while the expression of adiponectin was inhibited (Figure 5, C), under chronic restraint stress. For the PVN activation paradigm, the serum CORT level was unchanged, while the expression of adiponectin unchanged in eWAT (Figure 5, G lower), even increased in iWAT (Figure 5, G upper). Taken together, these data indicated that increased CORT level played a crucial role in the downregulation of adiponectin under chronic restraint stress.

Reference:

1. Kaikaew K, Steenbergen J, van Dijk TH, Grefhorst A, Visser JA (2019) Sex Difference in Corticosterone-Induced Insulin Resistance in Mice. *Endocrinology* 160(10):2367-2387.

2. Dang TQ, Yoon N, Chasiotis H, Dunford EC, Feng Q, He P, Riddell MC, Kelly SP, Sweeney G (2017) Transendothelial movement of adiponectin is restricted by glucocorticoids. *J Endocrinol* 234(2):101-114.

Minor issues:

1. In all of the figures, pictures of the brains should be at higher magnification and include landmarks of the areas of interest. As presented, it is hard to see colocalization of the markers being tested.

We are deeply thankful for the suggestions. We added landmarks for the brain areas of interest in all figures (Fig 2, Fig 3 and EV1). For the colocalization of PRV and c-fos in PVN, both low and high magnification of photomicrograph were shown in the Fig EV2.

2. For the pseudorabies tracing experiments, it is unclear how many synapses are transited by the virus before it reaches the PVN. The timing after infection is critical, and maybe the authors could show a time course that would indicate the number of synapses between the PVN and adipose tissue.

We are deeply thankful for the suggestions. We performed retrograde tracing to show the time course of infection by PRV (Fig EV1).

3. In the description of the chronic dreadd activation experiment, it is unclear when CNO was injected to chronically activate/inhibit PVN neurons. Was CNO given once per day for each of 42 days?

We thank the reviewer for raising these important points. For chronical activation/inhibition of PVN neurons, CNO was intraperitoneally injected once every 2 days for 42 days (Page 6, line 163-164).

4. The manuscript should be edited for English usage. Although it is clearly written, there are many grammatical errors.

We are grateful to the reviewer by this suggestion. The flow and writing of our manuscript have been improved with assistance from native English speakers.

Referee #2:

In this manuscript, the Authors explore the role of PVN-sympathetic-adipose circuit in depression and insulin resistance under chronic stress. The Authors note that chronic restraint stress increases sympathetic innervation in white adipose tissue (WAT) and is associated with impaired glucose and insulin

metabolism as well as depressive-like behaviors. The Authors also determine that restraint stress activates neurons in the PVN and that chemogenetic activation of the PVN is sufficient to upregulate sympathetic innervation in WAT. Given that sympathetic innervation activates β3-adrenergic receptor signaling in adipocytes, the Authors further show that pharmacological blockade of the β3-adrenergic receptor ameliorates depressive-like behaviors and insulin metabolism in a chronic restraint model. The Authors also characterize the expression of several adipokines in the chronic restraint and PVN activation models and further explore their transcriptional regulation.

The manuscript under review addresses a timely topic and represents a welcome addition to the literature linking chronic stress with depression and metabolic disorders. The manuscript is, therefore, of potential interest to a broad readership, ranging from the fields of metabolism to neuroscience and physiology.

Given the interesting nature of the topic at hand, it is my opinion that the Authors could improve several aspects of this manuscript:

• In Figure 2B, the Authors show colocalization of neurons traced from the white adipose tissue (WAT) with neurons activated (cFos positive) by restraint stress. It would be important to show whether this experimental model induces any neuronal activation even without exposure to stress. PRV tracing plus cFos staining in control mice would be informative.

We thank the reviewer for raising these important points. Following the reviewer's instruction, we have performed experiment to address this issue (Fig EV2).

• In Figure 2D, the experimental details are not clear. The Authors should clarify whether the CNO injection was performed just one time or daily for 42 days. Along the same line, in Figure 2G and 2H the experimental details are not sufficiently explained. It is not clear whether the experimental mice were injected with the CNO during the additional 6 days of restraint stress or not. It is also not obvious to what procedures the control mice were subjected. Similarly, in Figure 2K and 2L, it is not clear whether the GTT and ITT was performed without the additional restraint stress regimen or nor. It is difficult to interpret the results without confirming the experimental details.

We thank the reviewer for raising these important points. For Figure 2D, CNO was intraperitoneally injected once every 2 days for 42 days (Page 6, line 163-164). In Figure 2G-2J, the experimental mice weren't injected with the CNO during the additional 6 days of restraint stress (Page 7, line 175-179). In Figure 2K and 2L, the GTT and ITT was performed without the additional restraint stress regimen (Page 7, line 179-180).

• L748337 is a β3-adrenergic receptor antagonist and this information could be added to the manuscript. In Figure 3, the Authors could explain better the experimental details. It is not clear whether the L748337 was administered daily and to what procedures the control mice were subjected.

We are grateful to the reviewer by this suggestion. The information that L748337 is a β3-adrenergic receptor antagonist had been added to the revised manuscript (Page 8, line 214-216). The Restraint+L748337 group mice received L748337 administration daily during the chronic restraint treatment (Page 8, line 214-215), while both control and restraint groups were injected same volume phosphate buffered saline (PBS).

• In the sub-chapter "Regulation of adipokines related to depression and insulin resistance under chronic restraint stress", the Authors conclude: "Taken together, the findings suggested that decreased levels of adipokines, including leptin, adiponectin, Angptl4 and Sfrp5, may account for the depressive-like behaviours and insulin resistance induced by chronic stress or chronic activation of PVN neurons". While this correlation seems to be correct for the observed WAT changes in chronic restraint stress model, it is not entirely accurate for the PVN activation. The adiponectin levels in WAT (both gene expression and protein) where increased in mice expressing activatory Dreadds in the PVN. The Authors should acknowledge and discuss these discrepancies.

We are deeply thankful for the critiques and suggestions. In our revised manuscript, we acknowledged and discuss these discrepancies mentioned above (Page 9, line240-252). As the HPA axis also has a role in regulating insulin resistance and depression, we assayed the HPA activity under both chronic restraint stress and PVN activation paradigms (Fig EV5). Our results

showed that chronic restraint treatment increased the serum CORT level (Fig EV5E), while chronical activation of PVN didn't affect the serum CORT level (Fig EV5J). Previous studies indicated that CORT inhibited the expression of adiponectin[1,2]. In our study, the serum CORT level increased (Fig EV5E), while the expression of adiponectin was inhibited (Fig 5C), under chronic restraint stress. For the PVN activation paradigm, the serum CORT level was unchanged (Fig EV5J), while the expression of adiponectin increased in iWAT (Fig 5G upper) and was unchanged in eWAT (Fig 5G lower). Taken together, these data indicated that increased CORT level played a crucial role in downregulation of adiponectin under chronic restraint stress.

Reference:

1. Kaikaew K, Steenbergen J, van Dijk TH, Grefhorst A, Visser JA (2019) Sex Difference in Corticosterone-Induced Insulin Resistance in Mice. *Endocrinology* 160(10):2367-2387.

2. Dang TQ, Yoon N, Chasiotis H, Dunford EC, Feng Q, He P, Riddell MC, Kelly SP, Sweeney G (2017) Transendothelial movement of adiponectin is restricted by glucocorticoids. *J Endocrinol* 234(2):101-114.

• It is well established that activation of the sympathetic outputs in WAT drives lipolysis. It would be interesting to see whether the lipolytic pathway in adipocytes and the FFA levels in the blood are affected in the chronic restraint stress and PVN activation models. The Authors show convincing images of WAT sympathetic innervation, but what happens to WAT histology and the

mass of WAT in both experimental models?

We thank the reviewer for raising these important points. Previous studies showed that acute activation of the sympathetic outputs in WAT induced lipolysis under cold exposure or fasting, in which leptin served an important role[3,4]. In the chronic restraint stress and PVN activation models, the serum leptin levels decreased (Fig 5B and 5F), while there were no significant difference in both the serum FFA levels and the mass of WAT (Fig EV3). Decreased serum leptin levels may counter the lipolysis of WAT induced by sympathetic activation.

Reference:

3. Wang P, Loh KH, Wu M, Morgan DA, Schneeberger M, Yu X, Chi J, Kosse C, Kim D, Rahmouni K, et al (2020) A leptin-BDNF pathway regulating sympathetic innervation of adipose tissue. *Nature* 583(7818):839-844.

4. Zeng W, Pirzgalska RM, Pereira MM, Kubasova N, Barateiro A, Seixas E, Lu YH, Kozlova A, Voss H, Martins GG, et al (2015) Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell* 163(1):84-94.

Other points on the accompanying material:

• In plots showing the RT-PCR data it is not clear how the relative gene expression was calculated.

The housekeeping gene β-tubulin was used as a reference gene for normalization of gene expression. The $2^{-\triangle\triangle Ct}$ method was used to calculate the relative gene expression[5] (Page21, line584-587).

Reference:

5. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.

• Figure legends should include information about the statistical analyses that were used.

We are grateful to the reviewer by this suggestion. We have added the information about the statistical analyses that were used in the Figure legends.

• In Figure 1A, the timeline should be labeled with "days"

• There is a typo in Figure 2J ("locomptor")

The Authors are encouraged to correct some grammar/text issues; examples:

• "Chronically activation of PVN neurons responding..."

• "These ChIP results may could explain

We thank the reviewer for pointing out these mistakes. All typos/grammar errors have been corrected in the revised manuscript.

Referee #3:

1. Overall, the manuscript could better benefit from a more elaborated explanation on how this chronic stress restraints translate into "depression" or a "depressive" state of the mice. The authors wanted to emphasize the transnationality of their study to human depressive states and insulin resistant and I am not quite sure if the model used, as well as de behavioral test

performed, are the most suitable ones. The authors can still point out on discussion the relation to depression and insulin resistance.

We are grateful to the reviewer by this suggestion. All overinterpretations are removed from the manuscript.

2. One limitation is that authors after the pseudorabies virus tracing experiment plus the cFos staining go directly to the PVH. I guess there should be also other brain regions receiving inputs from the SNS (such as DMH or VMH, as the authors pointed in the introduction), as well as other cFOS positive brain areas after the chronic restraint. Including more brain regions images with positive retrotracing or cFOS positive neurons, would increase the quality of the paper.

We are grateful to the reviewer by this suggestion. We performed retrograde tracing to show the time course of infection by pseudorabies virus (PRV) (Fig EV1). Indeed, there were cFOS positive brain regions under restraint which weren't traced by PRV (Fig EV4A-4F).

3. In accordance with the previous point, knowing other brains areas that are not involved in this restraint activation of neuronal populations (performing activating, or inhibiting neuronal experiments in these areas with the same viral approach than the PVH), would strengthen the conclusions.

We are deeply thankful for this suggestion. The paraventricular thalamic nucleus (PV) activated under restraint stress which wasn't labelled by pseudorabies virus on the 6th day (Fig EV4A-4F). Chronical activation of PV

with the same viral approach than the PVH didn't affect the sympathetic innervation in WAT (Fig EV4G-4L), and didn't induce depressive-like behaviours and insulin resistance (Fig EV4M-4R). These results highlighted the importance of PVH in the increased sympathetic innervation, depressive-like behaviours and insulin resistance induced by chronic restraint stress.

4. For GTT and ITT analysis, showing the area under the curve or performing HOMA-IR analysis would reinforce results.

We are grateful to the reviewer by this valuable advice. All the GTT and ITT analysis showed the area under the curve in our revised manuscript.

5. The PVH also receives inputs from the ARC, and it is also involved in the regulation of food intake. Could the authors also provide food intake data on their chronic restraint paradigm?

We are grateful to the reviewer by this suggestion. We have measured the amount of accumulative of 24h food intake after chronic restraint treatment (Fig EV3A).

6. It is also mentioned through the manuscript the HPA axis might also have a role in regulating chronic stress and depression. Could the authors provide some data on how is the state of this axis on their models? Have authors assayed CRH mRNA and/or protein levels in the PVH? Circulating ACTH or CORT levels?

We are deeply thankful for the suggestions. We have assayed the CRH mRNA and protein in the PVH, the serum ACTH and CORT levels (Fig EV5).

7. Do the authors have any guess on which neuronal population might be mediating the effects of chronic restraint within de PVH? In keeping with the former point, could CRH neurons be involved?

We thank the reviewer for raising these important points. Activation of HPA axis is a hallmark of the physiological reaction to stress. It is indicated that there are several neuron types in the PVH that respond to stress, including corticotropin releasing hormone (CRH) neurons which initiate HPA axis activity through releasing CRH. The end-hormones of HPA axis, corticosterone (CORT), is involved in the occurrence and development of depression and insulin resistance. The negative feedback effect of HPA activity is mediated by glucocorticoid receptors at all levels of the HPA axis[6,7]. Our results showed that chronic restraint treatment increased the serum CORT level (Fig EV5E) and decreased adrenocorticotropin (ACTH) (Fig EV5D), while the expression of CRH in the PVH was unchanged (Fig EV5A-5C). The expression of ACTH and CRH may be inhibited through the negative feedback loop of the HPA axis. Chronical activation of PVN didn't affect the serum CORT level (Fig EV5J). Previous studies indicated that CORT inhibited the expression of adiponectin[1,2]. In our study, the expression of adiponectin was inhibited (Fig 5C), under chronic restraint stress. For the PVN activation paradigm, the expression of adiponectin was unchanged in eWAT (Fig 5G lower), even

increased in iWAT (Fig 5G upper). These results indicated that increased CORT level played a crucial role in downregulation of adiponectin under chronic restraint stress. Taken together, these data suggested that both autonomic and neuroendocrine (CRH neurons) compartments of PVN were involved in mediating the effects of chronic restraint.

Reference:

1. Kaikaew K, Steenbergen J, van Dijk TH, Grefhorst A, Visser JA (2019) Sex Difference in Corticosterone-Induced Insulin Resistance in Mice. *Endocrinology* 160(10):2367-2387.

2. Dang TQ, Yoon N, Chasiotis H, Dunford EC, Feng Q, He P, Riddell MC, Kelly SP, Sweeney G (2017) Transendothelial movement of adiponectin is restricted by glucocorticoids. *J Endocrinol* 234(2):101-114.

6. Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, Scheimann J, Myers B (2016) Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. *Compr Physiol* 6(2):603-21.

7. Jiang Z, Rajamanickam S, Justice NJ (2019) CRF signaling between neurons in the paraventricular nucleus of the hypothalamus (PVN) coordinates stress responses. *Neurobiol Stress* 10;11:100192.

8. In the experimental paradigm of the β-antagonist, it is unclear the frequency of the treatment, please explain in the Methods or the Figure Legend. Also,

please indicate in Figure 5, if the stress paradigm is the same than the previous one, it is not really clear in the text.

We thank the reviewer for raising these important points. In Figure 4, the Restraint+L748337 group mice received L748337 administration each day during the chronic restraint treatment, while both Control and Restraint groups were injected same volume phosphate buffered saline (PBS). In Figure 5, the stress paradigm is the same than the previous one.

9. Could the authors provide some insight on why restraint induce a negative regulation of adiponectin (both at RNA and protein levels) but chronic activation of PVH neurons, induced the opposite effect?

We thank the reviewer for raising these important points. As the HPA axis might also have a role in regulating insulin resistance and depression, we assayed the HPA activity under both chronic restraint stress and PVH activation paradigms (Fig EV5). Previous studies indicated that CORT reduced the expression of adiponectin[1,2]. In our study, the serum CORT level increased (Fig EV5E), while the expression of adiponectin was inhibited (Fig 5C), under chronic restraint stress. For the PVH activation paradigm, the serum CORT level was unchanged (Fig EV5J), while the expression of adiponectin increased in iWAT (Fig 5G upper) and was unchanged in eWAT (Fig 5G lower). Taken together, these data indicated that increased CORT level played a crucial role in downregulation of adiponectin under chronic restraint stress.

Reference:

1. Kaikaew K, Steenbergen J, van Dijk TH, Grefhorst A, Visser JA (2019) Sex Difference in Corticosterone-Induced Insulin Resistance in Mice. *Endocrinology* 160(10):2367-2387.

2. Dang TQ, Yoon N, Chasiotis H, Dunford EC, Feng Q, He P, Riddell MC, Kelly SP, Sweeney G (2017) Transendothelial movement of adiponectin is restricted by glucocorticoids. *J Endocrinol* 234(2):101-114.

Dear Dr. Liu,

My colleague Esther is currently out of office. Therefore, I stepped in as the secondary handling editor of your manuscript.

Thank you for submitting your revised manuscript. It has now been seen by referees #1 and #3. Referee #1 also assessed the response to the initial concerns of referee #2.

As you can see, the referees find that the study is significantly improved during revision and recommends publication. However, I need you to address the points below before we can accept the manuscript.

- Please address the remaining concerns of referee #1.
- We note that there are currently 6 keywords, yet we can accommodate up to 5. Please remove one of the keywords.
- The Conflict of Interest section needs to be renamed as "Disclosure and competing interests statement"
- Author Contributions section needs to be removed from the manuscript.
- EMBO Press policy asks for all corresponding authors to link to their ORCID iDs. We note that the ORCID iD of Dr. Wei Li is currently missing. You can read about the change under "Authorship Guidelines" in the Guide to Authors here: https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

In order to link your ORCID iD to your account in our manuscript tracking system, please do the following:

1. Click the 'Modify Profile' link at the bottom of your homepage in our system.
2. On the next page you will see a box halfway down the page titled ORCID*. Below this box is red text reading 'To Register/Link to ORCID, click here'. Please follow that link: you will be taken to ORCID where you can log in to your account (or create an account if you don't have one)
3. You will then be asked to authorise Wiley to access your ORCID information. Once you have approved the linking, you will be brought back to our manuscript system.

We regret that we cannot do this linking on your behalf for security reasons.
- Please upload the source data for EV Figures as one folder.
- Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences (max 35 words) that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
- In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size should be 550 pixels wide and 300-600 pixels tall.
- Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Review of revised manuscript:
I have carefully reviewed the manuscript. The authors have made substantial improvements, primarily through the addition of figures that address the concerns that I had. In addition, the authors have provided clarifications and additions that address the comments of reviewer 2. I feel that the revised manuscript is now ready for publication. I think the manuscript could be improved by incorporating the Cort data into the main figures instead of putting it in the supplemental figures, as this is a critical piece of data that informs on the potential mechanisms by which the PVN influences gene transcription in WAT.
After looking at the supplemental figure including Cort measurements from plasma samples, it seems that the quantification is perhaps off by an order of magnitude. Cort normally circulates in the 30-50ng/ml range, and peaks in the 200-300ng/ml range. This should be recalculated and the figure should be corrected.

Referee #3:

All my comments have been addressed.

Referee #1:

I have carefully reviewed the manuscript. The authors have made substantial improvements, primarily through the addition of figures that address the concerns that I had. In addition, the authors have provided clarifications and additions that address the comments of reviewer 2. I feel that the revised manuscript is now ready for publication. I think the manuscript could be improved by incorporating the Cort data into the main figures instead of putting it in the supplemental figures, as this is a critical piece of data that informs on the potential mechanisms by which the PVN influences gene transcription in WAT.

After looking at the supplemental figure including Cort measurements from plasma samples, it seems that the quantification is perhaps off by an order of magnitude. Cort normally circulates in the 30-50ng/ml range, and peaks in the 200-300ng/ml range. This should be recalculated and the figure should be corrected.

We thank the reviewer for pointing out this mistake. We have retested the serum Cort using the original samples and same reagent (Fig EV 5E and 5J).

Referee #3:

All my comments have been addressed.

We are deeply thankful for your comments and suggestions.

Dear Dr. Liu,

Thank you for sending the revised ms files. It looks good now, except that I think that the abstract should be slightly rewritten. Do you agree with the following abstract, and please rewrite the one sentence where I left a comment:

Chronic stress induces depression and insulin resistance, between which there is a bi-directional relationship. However, the mechanisms underlying this comorbidity remain unclear. White adipose tissue (WAT), innervated by sympathetic nerves, serves as a central node in the interorgan crosstalk through adipokines. Abnormal secretion of adipokines is involved in mood disorders and metabolic morbidities. We describe here a brain-sympathetic nerve-adipose circuit originating in the hypothalamic paraventricular nucleus (PVN) with a role in depression and insulin resistance induced by chronic stress. A subset of PVN neurons are traced by pseudorabies virus (PRV) 'from WAT and activated by restraint' [the second half of the sentence is not clear. Please rewrite]. Chemogenetic manipulations suggest a role for the PVN in depression and insulin resistance under chronic stress. Chronic stress increases the sympathetic innervation of WAT and downregulates several antidepressant and insulin-sensitizing adipokines, including leptin, adiponectin, Angptl4 and Sfrp5, and chronic activation of the PVN has similar effects. β-adrenergic receptors translate sympathetic tone into an adipose response, inducing downregulation of those adipokines and depressive-like behaviours and insulin resistance. We finally show that AP-1 has a role in the regulation of adipokine expression under chronic stress.

I also made a few minor changes to the blurb and bullet points. Please let me know whether you agree with:

Under chronic stress, the PVN increases sympathetic innervation of adipose tissues and represses adipokine expression through AP-1, which contributes to the development of depression and insulin resistance.

1. Chronic activation of PVN neurons responding to restraint stress contributes to the development of depression and insulin resistance.
2. Chronic activation of PVN neurons responding to restraint stress increases sympathetic innervation of adipose tissues.
3. The β-adrenergic receptors translate sympathetic tone into the expression regulation of some adipokines, including leptin, adiponectin, Angptl4, Sfrp5 and Fbn1.
4. AP-1 proteins have a role in the regulation of adipokine expression by the PVN-sympathetic nerve pathway.

Regarding the synopsis image, the text in the image could be slightly larger, if possible. Also, is the cell in the lower right corner of the image a white adipose cell? If yes, please label the cell in the image.

We also noticed now that several of the figures of your ms are too long. All main and EV figures should fit onto one page and all text needs to be readable at the final size.

Please see our guidelines for figure preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

Regarding your figures we notice that :

Fig 2: some text is too small
Fig 4: some text is too small
Fig 5: figure is too long
Fig 6: some text is too small
Fig 7: figure is too long
Fig 8: figure is too long

EV1: is too long
EV4: too long

Can you please send us new figures? If you decide to split some of the figures into 2, please also send us a new, corrected manuscript file. I am sorry that we missed this before.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD

Senior Editor
EMBO reports

The authors have addressed all minor editorial requests.

Dr. Bin Liu
Binzhou Medical University Hospital
No. 661 Huanghe 2nd Road
Binzhou, Shandong 256603
China

Dear Dr. Liu,

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

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

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

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

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

Yours sincerely,

Esther Schnapp, PhD
Senior Editor
EMBO reports

****

THINGS TO DO NOW:

Please note that you will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/er_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

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

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

All further communications concerning your paper should quote reference number EMBOR-2023-57176V4 and be addressed to emboreports@wiley.com.

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

EMBO Press Author Checklist

Corresponding Author Name: BinLiu	**USEFUL LINKS FOR COMPLETING THIS FORM**
Journal Submitted to:EMBO Reports	The EMBO Journal - Author Guidelines
Manuscript Number: EMBOR-2023-57176	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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Materials

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

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

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

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
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.	Not Applicable	

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

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

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	

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	

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.	Yes	Materials and Methods
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	Materials and Methods， Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods
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? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods， Figures

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

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
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	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
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	

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	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
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	

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	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	