EMBO reports

N-acetyl-L-tyrosine is an intrinsic triggering factor of mitohormesis in stressed animals

Takashi Matsumura, Outa Uryu, Fumikazu Matsuhisa, Keiji Tajiri, Hitoshi Matsumoto, Yoichi Hayakawa

Review timeline:

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Editorial Decision:	29 October 2019
Revision received:	9 December 2019
Editorial Decision:	4 February 2020
Revision received:	5 February 2020
Accepted:	11 February 2020

Editor: Martina Rembold

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 29 October 2019

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, the referees also point out that it will require further experimental work to dissect how NAT triggers mitohormesis. In particular, the roles of ROS and the Nrf2-mediated anti-oxidant response need to be further substantiated. Please also remove the reference to the human preliminary experiment in the Discussion and provide proof of ethics authorization for the human plasma samples, if required. I could not find information on the source of the samples in the text. Please also note that all materials and methods must be part of the main manuscript and may not be in the Supplement.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) 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 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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 (only if relevant).

2) Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter blots in these cases. No statistics can 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).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

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

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

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<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>)

6) 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.

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

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

can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

9) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. 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.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

10) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File 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.

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

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

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REFEREE REPORTS

Referee #1:

The authors identify two acetylated amino acids, n-acetylcysteine (NAC) and N-acetyltyrosine (NAT), to potentially mediate a mitohormetic response and subsequently stress resistance in different animal models.
They mechanistically demonstrate for NAT that NRF2/KEAP1 and FoxO signalling contributes to this effect; also the NAT effect appears cell-autonomous since blocking amino-acid transport prevents the phenotype.
However, the proposed mechanism (Fig. 4A) and specifically the signaling role of ROS is insufficiently supported by the data presented. While the majority of findings was obtained with NAT, the authors thankfully and nevertheless show that the potent antioxidant and GSH precursor NAC exerts increased stress resistance. *IF* this is mediated by ROS the key question is: why does an antioxidant not interfere with the proposed ROS signal? The published body of evidence on ROS signaling and stress resistance in diverse organisms has repeatedly ahown that NAC blunts or completely abolishes ROS signals, and hence stress resistance. Also, ROS inducers like rotenone (complex I) and antimycin (complex III) increase stress resistance, while here antimycin has no effect and reduces the effectiveness of NAT.
While I am not claiming that this is scientifically impossible, it nevertheless is in conflict with evidence from several laboratories and hence needs to be further elucidated. Employing ROS quantification in cell and/or larvae, as well as analyses of the biochemical effect of NAT on mitochondria (as proposed) will hopefully help clarifying this.

Referee #2:

This is a very interesting and high novel research paper. The main finding is the identification of N-acetyl-tyrosine as a hormesis-mediating factor in cells and tissues from different species.
Some of the explorations of the roles of this substance in the various models used are partial, but this is acceptable in the context of a Report, especially given the fact that the data are overall consistent across models and accross species.
I have three specific recommendations:
1. In the Conclusions, please completely remove the reference to a human "preliminary experiment" on a "single case" for which the "data are not shown".
2. For the studies done with human serum, please provide documentation of proper ethics authorization (or exemption, as appropriate), as done for the mouse studies.
3. The functional importance of Keap1 mRNA induction is puzzling. Since N-acetyl-tyrosine induces Keap1 expression via Foxo, this might suppress the Nrf2 antioxidant response, which seems inconsistent with a hormetic setting where antioxidant genes regulated by Nrf2 such as SOD are actually upregulated as shown in the manuscript. Alternatively, since Keap1 is itself a Nrf2 target gene, the Nrf2 antioxidant response may be activated by N-acetyl-tyrosine. Please dissect further the activation status of the Nrf2 pathway in response to N-acetyl-cystein treatment to elucidate which of these two scenarios is true (is it activated or inhibited?). I would suggest to address this by in vivo experiments in Drosophila.

1st Revision - authors' response 9 December 2019

Referee #1

Comments:
The authors identify two acetylated amino acids, n-acetylcysteine (NAC) and N-acetyltyrosine (NAT), to potentially mediate a mitohormetic response and subsequently stress resistance in different animal models.
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*However, the proposed mechanism (Fig. 4A) and specifically the signaling role of ROS is insufficiently supported by the data presented. While the majority of findings was obtained with NAT, the authors thankfully and nevertheless show that the potent antioxidant and GSH precursor NAC exerts increased stress resistance. *IF* this is mediated by ROS the key question is: why does an antioxidant not interfere with the proposed ROS signal? The published body of evidence on ROS signaling and stress resistance in diverse organisms has repeatedly ahown that NAC blunts or completely abolishes ROS signals, and hence stress resistance. Also, ROS inducers like rotenone (complex I) and antimycin (complex III) increase stress resistance, while here antimycin has no effect and reduces the effectiveness of NAT.*
While I am not claiming that this is scientifically impossible, it nevertheless is in conflict with evidence from several laboratories and hence needs to be further elucidated. Employing ROS quantification in cell and/or larvae, as well as analyses of the biochemical effect of NAT on mitochondria (as proposed) will hopefully help clarifying this.

As the referee notes, N-acetylcysteine (NAC) is generally known as a pharmacological antioxidant. Although it is often assumed that NAC scavenges oxidants directly through its thiol group, this has been reported to be very unlikely in the light of kinetic data because the rate constants for the reaction of NAC with relevant physiological oxidants such as H_2O_2 and $O_2^{\cdot-}$ are too low (0.16 and 68 $M^{-1}s^{-1}$, respectively) to make a significant contribution to oxidant scavenging (Benrahmoune et al., Free Radic. Biol. Med., 29, 775-782 (2000)), indicating that NAC itself is a poor scavenger of oxidants. In fact, we confirmed that N-acetyltyrosine (NAT) showed slightly higher antioxidant activity than NAC when they were mixed with H_2O_2,as shown in the attached graph Fig. A. Therefore, the antioxidant properties of NAC may be ascribed rather to NAC-derived products such as glutathione and hydrogen sulfide. Recently, sulfane sulfur species produced from hydrogen sulfide exert the antioxidative and cytoprotective effects provided by NAC (Ezerina et al., Cell Chem. Biol. 25, 447-459 (2018)). Based on the information, we have done experiments to examine effects of NAC on mitochondria following the suggestion of this referee. The results clearly showed the similarity between NAC and NAT in their effects on mitochondria: NAC transiently induced depolarization of mitochondria following elevation of mROS concentrations in S2 cells. These data have been added as supplementary data (Appendix Figure S13) together with the explanation in the Results and Discussion. Furthermore, we examined the effects of low concentrations of antimycin A and rotenone to S2 cells and demonstrated that pretreatment by both chemicals with low concentrations (around one-thousandth of the inhibitory concentrations) induced slightly but

significantly elevated survivals of S2 cells after heat stress, as shown in attached graph Fig. B. Although we think that the results must be interesting, it is possible that these data confuse readers because they are not directly related to the main focus of this paper. Therefore, we have not included these data (attached graph Fig. B as well as Fig. A) in this revised manuscript.

As suggested by this referee, many conflicting results have been reported concerning the physiological effects of certain chemicals including antioxidants and respiratory poisons. However, our present study indicates that the timing of ROS measurements as well as concentrations of test chemicals are critically important in bringing our experiments to a correct conclusion.

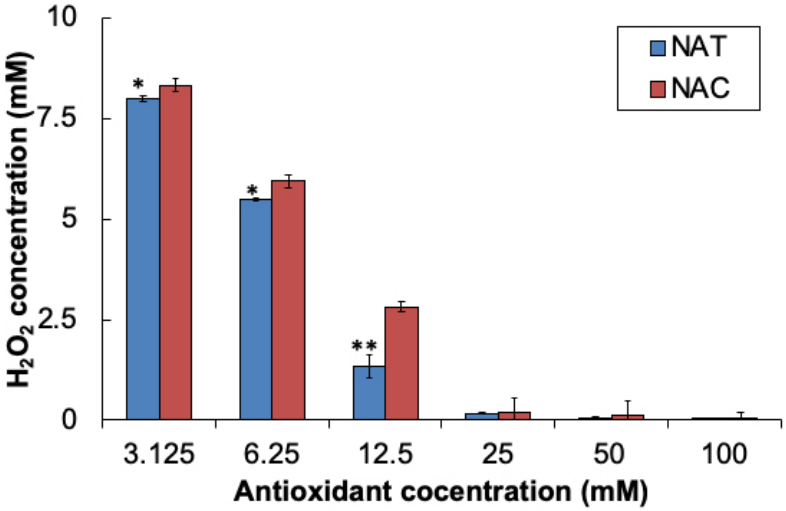

Fig. A: H_2O_2 concentrations 30 min after mixing N-acetyltyrosine (NAT) or N-acetylcysteine (NAC) with 8.8 mM H_2O_2 in PBS at 25°C (data are means ± SEM; n=5). *P<0.05, **P<0.01 vs. NAC.

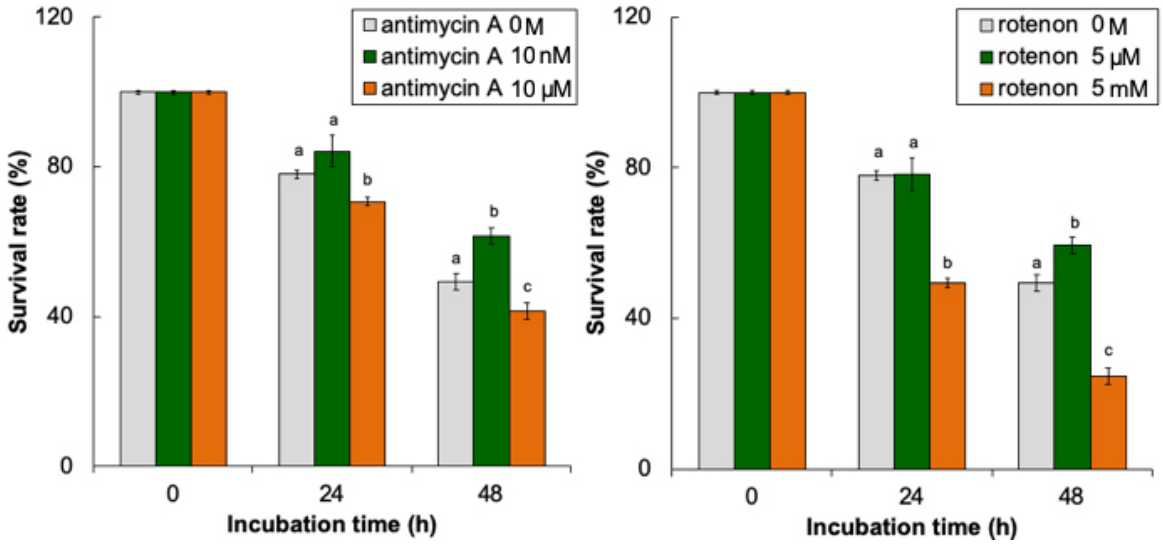

Fig. B: Pretreatment with low concentrations of antimycin A (left) and rotenone (right) elevates survivals of S2 cells after heat stress at 42°C for 60 min (data are means ± SEM; n=5). After pretreatment with chemicals for 12 h, those were removed by changing the medium and S2 cells were exposed to heat stress. Different letters above bars represent significant differences [P <0.05 (data are means ± SEM, n = 6)]

Referee #2:

Comments:
This is a very interesting and high novel research paper. The main finding is the identification of N-acetyl-tyrosine as a hormesis-mediating factor in cells and tissues from different species.
Some of the explorations of the roles of this substance in the various models used are partial, but this is acceptable in the context of a Report, especially given the fact that the data are overall consistent across models and accross species.
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1. We accept the referee's suggestion and have eliminated the reference to the human preliminary experiment.
2. The identification of human serum NAT by HPLC (Fig. 2A) was done using pooled human serum available for purchase, as described in the figure legend. Therefore, we believe that we do not need the special document concerning ethics authorization.
3. As the referee suggested, the mechanism by which NAT induces mitohormesis is quite complicated. Although we have not yet completely solved the puzzle, we presume the contribution of FoxO and Keap1-Nrf2 in this signaling system as follows. As we propose in the revised graphic depicting it (Fig. 4E), NAT must disturb mitochondrial redox status to induce ROS release. The NAT-induced activation of the FoxO-Keap1 signaling axis negatively regulates Nrf2 function and enforces ROS release from mitochondria because Nrf2 regulates mitochondrial homeostasis (Dinkova-Kostova and Abramov, Free Radic. Bol. Med., 88, 179-188 (2015)). However, this inhibition must be removed soon after ROS is released from mitochondria by losing the prominent regulator Nrf2, which allows Nrf2 to evade Keap1-mediated repression because ROS-induced Keap1 conformational changes liberate Nrf2 (Suzuki et al., Cell Rep. 28, 746-758 (2019)). Therefore, it is reasonable to presume that both FoxO and Nrf2 sequentially stimulate expression of their target genes with anti-stress abilities because NAT-induced elevation of *Keap1* expression would not cause long-lasting inhibition of Nrf2. To confirm this interpretation, we have some experiments using *in vivo RNAi* experiments. As we expected, neither *Keap1 RNAi nor Nrf2 RNAi Drosophila* larvae showed NAT-induced thermotolerance. These data have been added as supplementary data (Appendix Figure S14) together with the related explanation in the Results and Discussion because these at least partly support the above interpretation. As we mentioned above, we have slightly revised the graphic model by including Nrf2 (new Fig. 4E).

2nd Editorial Decision 4 February 2020

Thank you for the submission of your revised manuscript to EMBO reports. I apologize for the delay in handling your manuscript but we have only recently received the full set of referee reports that is copied below.

As you will see, both referees are very positive about the study and support publication after a minor revision. Referee 1 suggests to include the experiments currently only shown to the referees into the manuscript's Appendix.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

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REFEREE REPORTS

Referee #1:

The authors have addressed my concerns to a wide extent.
However, the additional experiments performed are only provided in comments to reviewers. These, however, should be included into the Supplement of the revised version.

Referee #2:

My comments on the first revision round have been well addressed. I have no further comments.

2nd Revision - authors' response 5 February 2020

The authors performed all minor editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Yoichi Hayakawa
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2019-49211

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We stated the detailes in the Figure legends and Materials and Methods. However, No statistical methods were used to predetermine sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	No statistical methods were used to predetermine sample size. Sample sizes were determined by magnitude and consistency of measurable differences. The precise number of animals used were indicated in the Figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data 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.	No there were not.
For animal studies, include a statement about randomization even if no randomization was used.	Mice used in the present study were randomly assigned to experimental groups but we do not state it.
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.	No there were not.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No we do not because investigators were not blinded during group allocation and data analysis.
5. For every figure, are statistical tests justified as appropriate?	We stated the detailes in the Figure legends and Materials and Methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We stated the detailes in the Materials and Methods.
Is there an estimate of variation within each group of data?	Yes there is.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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http://www.consort-statement.org/checklists/view/32-consort/66-title

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

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

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

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We state the detailed information of the antibody we used and include the references in the text.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used HCT116 cells (catalogue no:91091005, European Collection of Authenticated Cell Cultures (ECACC)). Cells were tested for no mycoplasma contamination before the experiment.

* 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.	Yes we state them.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	As we state in the Materials and Methods: all experiments on mice were conducted with the approval of Saga University Animal Care and Use Committee according to the National Institutes of Health guidelines.
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.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	We used only pooled human seruma vailable for purchase (#12181201, Cosmo Bio Co., Japan) in this study.
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.	This study is not related to human subject.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	This study is not related to human subject.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	This study is not related to human subject.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	This study is not related to human subject.
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.	This study is not related to human subject.
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.	This study is not related to human subject.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	This study contains no such data.
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).	This study contains no such data.
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).	This study contains no such data.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	This study contains no such data.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No we did not this time.