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The mitochondrial outer membrane protein MDI promotes local protein synthesis and mtDNA replication

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Transaction Report:

PLEASE NOTE that this manuscript was transferred from a different journal and the independent expert assessing suitability for The EMBO Journal had access to both the original anonymous comments as well as the point-by-point response provided by the authors.

1st Editorial Decision 19 September 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have considered the previous referee comments and as we discussed, we obtained the advice of an independent expert whose comments are shown below.

It is clear from the input of all four experts, that there is significant basic interest in the results of your screen. It is also clear that the mtDNA selection aspect of the manuscript is currently not compelling and should be removed from the manuscripts (including any related claims).

Our expert also sees fundamental limitations in the data supporting your claim for a direct link to protein synthesis (please see comments below). The expert suggests to attempt polysome profiling experiments to buttress this link.
For publication in the EMBO Journal it is in our view important to provide compelling support for this prominent claim in the paper. We appreciate that the polysome profiling experiment suggested is involved and its outcome uncertain. We are requesting it on the advice of our expert as we agree with him/her that it this experiment would go some way to support the claims made in your current title and abstract. There may of course be alternative experiments that you may wish to discuss, and we are open to your suggestions.

If you cannot or prefer not to develop the dataset, but are prepared to engage in the necessary toning down of the claims, we would nevertheless invite further discussion about potential publication in a sister journal of the EMBO Journal, as your screen and dataset is clearly of intrinsic interest and value as also underlined by the expert we consuted.

I would like to invite you to submit a revised version of the manuscript, addressing the comments of all the reviewers in detail (that include the previous referees forwarded to us - that is to pursue the revision as outlined in the 'revision plan' you had sent). I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

Referee #1 *(independent expert):*

'I have looked over this intriguing paper, the associated reviewers comments and the authors response. In my view there are some fascinating obvservations here but I'm not sure it all holds up as it is. Second, there looks to be two papers in here - the characterisation of the function of MDI/LARP1 and then their potential role in mtDNA selection. I would urge the authors to remove this data from the first manuscript, as I found it distracting, but ask them to then expand the work on the putative selectivity for the second paper.

Regarding the former, I am not convinced about the explanation that MDI/LARP directly affect synthesis of mitochondrially destined polypeptides, some of which could be involved in mtDNA replication. Its an interesting hypothesis but is just that. What is definitely intriguing is that loss of a mitochondrial outermembrane protein seems to result in loss of mtDNA during oocyte maturation. This sort of reminds me of a paper by Meeusen and Nunnari who showed in yeast that the mitochondrial matrix-localised nucleoid is part of a structure that spans both membranes (see attached). Of course, there is also the well documented association in protozoa of association between the kinetoplast and the flagellar basal body. Perhaps this protein is part of a structure in the ovariole that anchors the nucleoid ?

Why am I not immediately convinced by the protein synthesis hypothesis ? It is well known that in mammalian cells, mitochondria lacking mtDNA (rho0) or indeed in cells depleted for certain components of the multi-subunit respiratory or mitoribosomal complexes, that other components disappear or are markedly affected at steady state level. This is not that surprising and it has been generally believed that this is due to rapid proteolysis of other components when the complex itself doesn't assemble. Of course, if there is a deficit of mtDNA, as would be the case here, there would be only a limited amount of mitoribosomal rRNA (encoded by the mtDNA) and consequently a lack of assembled mitoribosome and a consequent loss of many steady state components of the mitoribosome. It has been accepted this is likely to be due to increased proteolysis in the matrix but never formally shown. Could this be due to a decrease in synthesis of some mRNAs that encode mitoribosomal components that are bound to cytosolic ribosomes attached to the mitochondrial outer membrane ? Perhaps, but this hasn't ever been shown, either. Irrespective, decreased mtDNA will lead to a decrease in steady state levels of mitoribosomal components and many other mitochondrial components involved in mtDNA expression, particularly components of the OXPHOS complexes. This is what the authors find in this paper, particularly on their proteome work and is just consistent with the lack of mtDNA. It would indeed be a remarkable finding if the authors were to show that this was due to the loss of synthesis of some mitochondrially destined components but there is quite a way to go to show this.

How could this be demonstrated ? The standard method for showing that mRNA is localised to the ribosome but is not translated would be to attempt polysome profiles and show the mRNA of interest moves towards the monosome and away from the polysome fractions. Would this be possible from such small amts of material ? The fractionation could be quite crude ie.make libraries of mRNA found in low density and high density fraction after density gradient centrifugation and compare ratios for candidate mRNAs. This would be convincing.

Finally, I just wonder whether this work could be publishable even in the absence of convincing data to explain how MDI/LARP is working ? The siRNA library screen is not really touched on. Finding a hit like this is impressive and there doesn't appear to be any doubt that depletion of these proteins

are leading to mtDNA depletion, which is a big surprise. In addition they have the expression of the mutated proteins to try and narrow down the important functional domains. There could be a small section to suggest that the function may be via MDI-LARP mediated protein synthesis at the surface but I would have major concerns about including this claim in the title as the data stands.'

1st Revision - authors' response 07 December 2015

Referee #1 (independent expert):

I have looked over this intriguing paper, the associated reviewers comments and the authors response. In my view there are some fascinating observations here but I'm not sure it all holds up as it is. Second, there looks to be two papers in here - the characterization of the function of MDI/LARP1 and then their potential role in mtDNA selection. I would urge the authors to remove this data from the first manuscript, as I found it distracting, but ask them to then expand the work on the putative selectivity for the second paper.

We took out the data on selective inheritance, revised the main text and reformatted Figure 2 accordingly in the revised manuscript.

Regarding the former, I am not convinced about the explanation that MDI/LARP directly affect synthesis of mitochondrially destined polypeptides, some of which could be involved in mtDNA replication. Its an interesting hypothesis but is just that. What is definitely intriguing is that loss of a mitochondrial outermembrane protein seems to result in loss of mtDNA during oocyte maturation. This sort of reminds me of a paper by Meeusen and Nunnari who showed in yeast that the mitochondrial matrix-localised nucleoid is part of a structure that spans both membranes (see attached). Of course, there is also the well documented association in protozoa of association between the kinetoplast and the flagellar basal body. Perhaps this protein is part of a structure in the ovariole that anchors the nucleoid?

We were aware of the earlier work in yeast by Nunnari group. In fact, we once considered this intriguing idea that MDI might anchor the nucleoid to a two-membrane spanning site as demonstrated in yeast. However, the *mdi* mutation affects mtDNA replication in the ovary only, and the only observed phenotype is female sterility. This argues against MDI as a ubiquitous nucleoid-associated protein that would be essential for mtDNA maintenance in all tissues and cells. More importantly, the Tom20-Larp fusion protein that constitutively localizes on the mitochondrial surface rescues mtDNA level and female fertility in the *mdi* mutant background. Thus the putative association of MDI to the nucleoid may not be crucial to the role of MDI/Larp in mtDNA replication during oogenesis. Based on our data, MDI's main function remains to recruit Larp to mitochondrial surface to promote local protein synthesis.
Therefore, even if MDI indeed associated with nucleoid, the physiological significance of this association remains to be explored. Nonetheless, we agree with the referee that this intriguing idea should be discussed. We elaborated on this aspect in our discussion, on page 23, line 5-19 in the revised manuscript.

Why am I not immediately convinced by the protein synthesis hypothesis? It is well known that in mammalian cells, mitochondria lacking mtDNA (rho0) or indeed in cells depleted for certain components of the multi-subunit respiratory or mitoribosomal complexes, that other components disappear or are markedly affected at steady state level. This is not that surprising and it has been generally believed that this is due to rapid proteolysis of other components when the complex itself doesn't assemble. Of course, if there is a deficit of mtDNA, as would be the case here, there would be only a limited amount of mitoribosomal rRNA (encoded by the mtDNA) and consequently a lack of assembled mitoribosome and a consequent loss of many steady state components of the mitoribosome. It has been accepted this is likely to be due to increased proteolysis in the matrix but never formally shown. Could this be due to a decrease in synthesis of some mRNAs that encode mitoribosomal components that are bound to cytosolic ribosomes attached to the mitochondrial outer membrane? Perhaps, but this hasn't ever been shown, either. Irrespective, decreased mtDNA will lead to a decrease in steady state levels of mitoribosomal components and many other mitochondrial components involved in mtDNA expression,

particularly components of the OXPHOS complexes. This is what the authors find in this paper, particularly on their proteome work and is just consistent with the lack of mtDNA. It would indeed be a remarkable finding if the authors were to show that this was due to the loss of synthesis of some mitochondrially destined components but there is quite a way to go to show this.

We now have new data including polysome profiling and pulse-chase labeling (please refer to our responses blow for detail), showing that the *de novo* synthesis of several candidates including Tamas, TFAM, mRPL19 and Cox4 is indeed reduced. Nonetheless, we appreciate the referee's insight. In fact, we think that the lack of mtDNA and the lack of assembled mitochondrial ribosomes might constitute a feed-forward loop to further exacerbate the defects in *mdi* mutant ovary, and hence acknowledge this aspect on page 18, line 2-6 in the revision.

How could this be demonstrated ? The standard method for showing that mRNA is localised to the ribosome but is not translated would be to attempt polysome profiles and show the mRNA of interest moves towards the monosome and away from the polysome fractions. Would this be possible from such small amts of material ? The fractionation could be quite crude ie.make libraries of mRNA found in low density and high density fraction after density gradient centrifugation and compare ratios for candidate mRNAs. This would be convincing.

We thank the referee for the suggestion. We have performed polysome profiling on several candidate genes. We found that the mRNAs of tamas, tfam, cox4 and mRPL19 were indeed reduced in the polysome fraction in MDI ovary, suggesting a reduced *de novo* synthesis of these proteins. We present the data in revised Fig 6D & 6E.

Finally, I just wonder whether this work could be publishable even in the absence of convincing data to explain how MDI/LARP is working? The siRNA library screen is not really touched on. Finding a hit like this is impressive and there doesn't appear to be any doubt that depletion of these proteins are leading to mtDNA depletion, which is a big surprise. In addition they have the expression of the mutated proteins to try and narrow down the important functional domains. There could be a small section to suggest that the function may be via MDI-LARP mediated protein synthesis at the surface but I would have major concerns about including this claim in the title as the data stands.'

We appreciate the referee's enthusiasm toward our work and specific suggestions to improve the manuscript. We have performed additional experiments as per referee's and other reviewers' suggestions. We now have additional evidence further substantiating the point that MDI-Larp promotes the synthesis of a subset of mitochondrial proteins, which is essential for mtDNA replication in ovary.
mdi gene was identified from an on-going RNAi screen, which has not been completed yet. Most identified hits have not been validated yet. We thus choose not to publish our screen.

2nd Editorial Decision 17 January 2016

Thank you for resubmitting your revised manuscript. It has now been seen again by our arbitrating expert, and his/her comments are enclosed. As you will see, the referee continues to support your manuscript and is broadly in favour of publication, pending satisfactory minor revision.

We will be happy, in principle, to publish your manuscript, contingent on your addressing the textural issues outlined below. We would also ask to reconsider whether it would not make sense to include some or all of the screen in this paper, as it will attract significantly more interest to the paper as a community resource. We understand that the referee is not insisting on this and neither are we, but we would like as much as you to ensure the manuscript receives the attention it deserves.

We would like to invite you to revise the manuscript texturally to address the important issue raised by our arbitrating referee 4 regarding the interpretation of the data. We agree with the referee that a

careful interpretation is warranted in this case as the mechanism of action of MDI remains ill defined, even though the effects described compelling.
Please therefore revise the main manuscript, but also the title and abstract accordingly.

We may consult further with the advisor if necessary.

Please integrate the Materials and Methods and essential references from the supplementary references list into the main paper (references are only captured by search and bibliographic databases in the main manuscript).

Please integrate a subset of the supplementary figures into the main manuscript as 'expanded view' figures labeled 'EV1, 2, etc.'. Details can be found the 'guide to authors'. The current supplementary figures are rather short/fragmented - please consider integrating panels into a smaller number of EV figures or add panels to existing main figures in the manuscript in as far as possible without disrupting the logic. If possible, this should include Table S4 (i.e. Table EV1).

The Appendix is labelled incorrectly throughout (it is correct in the manuscript call-outs). Supplementary tables should be included in the Appendix PDF.

We require a synopsis/standfirst: Please supply 4-5 bullet point that concisely summarize key conclusions of your study in a manner that is complementary to the abstract. Please preface this with a 30-40 word standfirst text, which should be complementary to the title. If possible, please supply an image to be used as a 'visual abstract'.

Thanks you for supplying the author checklist, which we have analyzed and accept.

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

As a matter of policy, competing manuscripts published during revision will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

Referee #1:

Many thanks for sending me the update. I believe the authors have done a solid job of accommodating the comments from all 3 reviewers. It has now lost the mtDNA segregation work, which was extremely confusing and they have cleared up their grammar as requested. They have also performed several new and quite challenging expts. Overall, I feel the paper should be published, although I am not convinced about their final hypothesis. There is no doubt that the screen has identified MDI as important for promoting the increase in mtDNA copy number. LARP has been implicated as an important mediator of mtDNA copy number maintenance and the expt to show that localisation of LARP to the mito outer membrane by fusion to TOM20 can rescue the mtDNA depletion is really compelling. Exactly what the molecular explanation for the phenotype is still unclear to me. It is clear that the steady state levels of the mtDNA polymerase, TAMAS, is decreased in the mdi mutant. This in itself is likely sufficient to explain the mtDNA depletion. The authors have now shown by polysome analysis that various mt transcripts have been shifted slightly towards the monosome and away from the polysome, consistent with a slight decrease in protein synthesis, but the authors claim it is localisation of the mRNA to the outer membrane that has been lost in the mdi mutant so why would there be a decrease in synthesis ? The proteomics data suggests a lot of OXPHOS and mitoribosome components are decreased in the MDI mutant eggs. This is

entirely consistent with the loss of mtDNA, resulting in decrease in mtDNA encoded mitoribosomal RNA and consequent loss of mtDNA-encoded OXPHOS components, without which the steady state levels of many mitoribosomal subunits and OXPHOS subunits would be depleted. I don't think any of us know why the depletion of MDI is leading to mtDNA depletion but the hypothesis that is suggested by the authors seems a reasonable (albeit not totally convincing) one. Bottom line - I think it a really interesting paper and should be published although there are still certainly questions.

Regarding the authors decision not to include the screen - whilst I think it would be great for the field if they did release these results, I can see why they want to hold on to this information and perhaps perform other expts to validate these hits before they publish. They have already carried out extensive tests to validate and characterise this one, MDI, mutation that they have identified by this screen and I think that is sufficient for this paper.

2nd Revision - authors' response 03 February 2016

We would like to thank the referee for supporting our manuscript. We have made a few textural revisions to address the referee's concerns as detailed below:

Overall, I feel the paper should be published, although I am not convinced about their final hypothesis. There is no doubt that the screen has identified MDI as important for promoting the increase in mtDNA copy number. LARP has been implicated as an important mediator of mtDNA copy number maintenance and the expt to show that localisation of LARP to the mito outer membrane by fusion to TOM20 can rescue the mtDNA depletion is really compelling. Exactly what the molecular explanation for the phenotype is still unclear to me. It is clear that the steady state levels of the mtDNA polymerase, TAMAS, is decreased in the mdi mutant. This in itself is likely sufficient to explain the mtDNA depletion.

Constitutively targeting Larp to mitochondria can rescue mtDNA deficiency and Larp promotes protein translation. It strongly suggests that MDI promotes mtDNA replication through boosting the local protein synthesis. Nonetheless, we agree with the referee that our model remains to be further verified. Therefore, we have toned down the claim in the revised manuscript.

We now change the title from "*The ~~mitochondrial~~ outer membrane protein MDI promotes local protein synthesis ~~essential for~~ mtDNA replication in the Drosophila ovary*" to "*The outer membrane protein MDI promotes local protein synthesis and mtDNA replication in the Drosophila ovary*"

We also revised the last sentence of the abstract on page 2, line15-18. Now it reads "*Our work suggests that a selective translational boost by the MDI-Larp complex on the outer mitochondrial membrane might be essential for mtDNA replication and mitochondrial biogenesis during oogenesis*".

Furthermore, we revised the first paragraph of the discussion on page 19, line 2-5. It now reads "*Here we demonstrate that MDI-Larp complex on mitochondrial surface promotes the translation of a subset of nuclear-encoded mitochondrial proteins and is required for mtDNA replication and mitochondrial biogenesis in Drosophila ovaries*".

but the authors claim it is localisation of the mRNA to the outer membrane that has been lost in the mdi mutant so why would there be a decrease in synthesis?

We have no data showing that the mRNA localization to outer membrane is lost in *mdi* mutant, nor we intend to argue for such claim. Nonetheless we rephrased two sentences in the discussion that presumably caused such confusion.

We revised the sentence on page 21, line 13-15. It now reads "*It is possible that local protein synthesis on mitochondrial surface mediated by MDI-Larp might relieve the translational inhibition by Nanos*".

We also revised the sentence on page 22, line 14-16. It now reads "*The locally synthesized proteins*

including mtDNA replication factors would be perfectly poised for efficient translocation into the mitochondria to drive massive mitochondrial biogenesis (Fig 7C)".

Regarding the authors decision not to include the screen - whilst I think it would be great for the field if they did release these results, I can see why they want to hold on to this information and perhaps perform other expts to validate these hits before they publish. They have already carried out extensive tests to validate and characterise this one, MDI, mutation that they have identified by this screen and I think that is sufficient for this paper.

Our RNAi screen has not been completed yet. We thus decide not to publish this preliminary data. We appreciate the referee's understanding and support for publication regardless of this data set.

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Corresponding Author Name: Hong Xu
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2015-92994

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 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.

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

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

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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?	N/A
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes are stated in the corresponding Figure legends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
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.	Yes. Flies for assessment were collected randomly from a large population.
For animal studies, include a statement about randomization even if no randomization was used.	Flies belongs to same experimental group used in the paper were collected randomly.
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5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes
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Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Page 15, Page S17
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page S15

* 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.	Page S13
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
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).	N/A
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22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

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