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ESCRT-mediated lysosome repair precedes lysophagy and promotes cell survival

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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 29th May 2018

Thank you for submitting your manuscript (EMBOJ-2018-99753) on a protective role for ESCRT proteins in lysosomal membrane repair and cell survival to The EMBO Journal. We have now received two referee reports on your study, which are enclosed below for your information.

As you can see, the referees appreciate the analysis also in light of the recent publication by Skowyra et al. However, they raise a number of different points. Referee #1 finds that the protective role of ESCRT proteins against lysosomal damage should be further investigated, e.g. by measuring plasma membrane integrity. Referee #2 points out that the effects of Alix on cell death needs to be discussed in greater detail. Finally, both referees request all the experiments to be properly quantified and statistically analyzed.

I have looked carefully at the comments and also discussed them with my colleagues. I agree with the referees that the issue of proper quantification and statistical analysis has to be addressed.

Regarding referee #1's major point: The suggested experiment is a good one and indeed such analysis would strengthen the paper. I don't know how straightforward it is to carry out plasma membrane integrity assays in your system. If the suggested experiment is feasible within a reasonable time frame then it would be good to include. If not, then let's discuss this point further. As said above, the added experiments would be great, but I also don't think that it is absolutely essential for the key message. In the absence of more definitive data supportive a protective role you would have to provide a more nuance discussion about this dataset. Please get back to me regarding this point.

REFEREE REPORTS:

Referee #1:

The manuscript by Radulovic and co-workers show a rapid recruitment of ESCRT machinery into damaged lysosomes, ESCRT-mediated re-establishment of lysosomal pH gradient and the necessity of this pathway for cell survival. The two first conclusions are fully in line with the recent Science paper by Skowyra et al demonstrating the role of ESCRT in lysosome repair, whereas the last one is a novel observation putatively of major importance to the field. The manuscript is well written, and experiments are well planned and performed, but proper quantification is lacking from the majority of figures. In addition to proper quantification and statistics, the cell death part requires some additional work as described below.

Main points
1. The only evidence provided for the protective effect of ESCRT against lysosome damage is a flow cytometry-based assay showing a larger portion of LLOMe-treated cells in sub-G1 fraction upon depletion of TSG10. Based on this assay the authors conclude that there is a dramatic increase in cell death, even though the data only shows an increase from approximately 8% to approximately 33%. Taking into consideration that all cells have lysosomal damage, this response does not appear dramatic when 2/3 of the cells, in fact, survive (or are not in sub-G1) the LLOMe treatment in the absence of ESCRT.
Sub-G1 cells have reduced DNA content and are generally considered as apoptotic. Yet, lysosomal damage does not necessarily induce apoptosis as demonstrated by the movie 7, which shows plasma membrane breakage. Thus, an assay measuring plasma membrane integrity would be more appropriate to use here and would probably reveal higher proportion of dying cells. Furthermore, proper dose responses and kinetics should be included to demonstrate the extent of cell death.

2. The authors conclude that also antihistamine-induced lysosomal damage triggers the recruitment of ESCRT. In order to conclude this, proper quantification and statistics should be applied. Furthermore, it would be important to also address whether ESCRT has a protective effect in the case of antihistamine-induced lysosomal damage.

3. Figures 1, 2, 3, 5 as well as many supplementary figures lack quantification and statistics. Figures 4 and 6 have some asterisks (very small font!) probably indicating significance, but what was compared and how is not indicated in the legend. All conclusions (also negative ones) should be supported by appropriate quantification and statistics!

Referee #2:

The manuscript by Radulovic and colleagues describes a role of ESCRT machinery on repair of damaged lysosome. Recently Skowyra reported essentially the same findings in Science. There are unique data in this paper showing that C. burnetti replication is enhanced by ESCRT-mediated lysosome repair, which add to the interest and novelty of the study. The findings are certainly important in the field, and it is a matter for the editors to decide how much the novelty has been compromised by the Skowyra publication. A number of big questions remain open in this area, most notably the proximal signal that triggers ESCRT recruitment to sites of lysosomal membrane damage, but it is not realistic to ask the authors to answer these in a revision.

Minor.

1. The authors indicate that subunits of ESCRT-I /-III but not ESCRT-0 or -II are recruited to damaged lysosomes upon LLOMe treatment (Fig. 2). In these experiments, the cells are treated with LLOMe for 2 h. Could ESCRT-0 or -II be recruited transiently and so missed in these experiments?
2. In Fig.4 A, the lysotracker foci are hard to distinguish. Please add high-resolution images.
3. In Fig.7, it seems that combined depletion of TSG101 and ALIX had a similar effect to depletion of TSG101 only, but depletion of ALIX also had some effect on cell death. Please discuss. Show the knockdown efficiency of siRNA oligos in this experiment.

1st Revision - authors' response 17th Aug 2018

Comments to editor:

In addition to the revisions based on the comments from the reviewers, the following changes have been made:
- The manuscript has been profoundly amended since the first submission. Data presented in Figures 1 and 2 were acquired with a different microscope in order to provide sharper images and clearer recruitment of ESCRTs upon endolysosomal membrane damage.
- Data on the ESCRT-II subunit VPS36 from Figure 2 were discarded as we were not able to determine if the tagged construct was fully functional. Instead we now provide new data on another ESCRT-II subunit, EAP30, is recruited (new Figure 2).
- In Figure EV3, data on CHMP3 were omitted due to heterogeneity of the cell line.
- In Figure 3, the graphs with siCHMP2A and siALIX were separated as experiments were repeated with lower siRNA concentration (20 nM siRNAs for ALIX).

Referee #1:

1. The only evidence provided for the protective effect of ESCRT against lysosome damage is a flow cytometry-based assay showing a larger portion of LLOMe-treated cells in sub-G1 fraction upon depletion of TSG10. Based on this assay the authors conclude that there is a dramatic increase in cell death, even though the data only shows an increase from approximately 8% to approximately 33%. Taking into consideration that all cells have lysosomal damage, this response does not appear dramatic when 2/3 of the cells, in fact, survive (or are not in sub-G1) the LLOMe treatment in the absence of ESCRT. Sub-G1 cells have reduced DNA content and are generally considered as apoptotic. Yet, lysosomal damage does not necessarily induce apoptosis as demonstrated by the movie 7, which shows plasma membrane breakage. Thus, an assay measuring plasma membrane integrity would be more appropriate to use here and would probably reveal higher proportion of dying cells. Furthermore, proper dose responses and kinetics should be included to demonstrate the extent of cell death.

We thank the reviewer for excellent comments, and we have now monitored plasma membrane rupture as suggested. We first investigated whether LLOMe compromises plasma membrane integrity and performed live-cell imaging with 250 µM LLOMe in the presence of 1 μg/ml propidium iodide (PI) (see Suppl.MovieX1 for reviewers). As shown before, LLOMe-induced endolysosomal membrane damage causes recruitment of CHMP4B to endolysosomal membranes, but does not lead to PI influx into the cells even after incubation times for up to 4 h. As a positive control, 0.05 % Triton-X100 was added to the cells at the end of the time-lapse acquisition. Triton-X100 leads to plasma membrane damage, PI influx and cell death. Next, following the same experimental setup as in Suppl.MovieX1 for reviewers, cells were depleted for TSG101 and ALIX and, when compared to the control cells (siCtrl), showed an increase in PI staining after induction of endolysosomal damage indicating compromised cell viability in the absence of ESCRTs (see Suppl.MovieX2 for reviewers). This indicates a protective role of ESCRTs upon endolysosomal damage, which is in agreement with our previous analyses.

In order to investigate cell viability and plasma membrane integrity in a quantitative way, we performed flow cytometry analyses of Annexin V and PI stained cells, as a standard procedure for the identification of early and late apoptotic and necrotic cells (see Figure 7 and EV5). In general, apoptotic cells (both early and late) were analyzed by measuring the externalization of phosphatidylserine on the plasma membrane using Annexin V, and necrosis was determined by measuring the permeability of the plasma membrane to PI [1]. To demonstrate the extent of cell death we included different time points after 3 h, 6 h and 10 h of treatment with lysosomotropic drug LLOMe. We chose a concentration of 250 μM LLOMe for most experiments, which resulted in clear recruitment of ESCRTs to damaged endolysosomes. Higher doses (up to 2.5 mM) were shown to induce autophagic clearance of damaged lysosomes [2] and may therefore confound conclusions on ESCRT-mediated repair and its influence on cell viability, making dose-response experiments difficult to interpret. As shown in Figure 7A, HeLa cells depleted for TSG101 and ALIX have elevated levels of Annexin V staining already after 3 h, and this is even more significant after 10 h of treatment with 250 µM LLOMe. Interestingly, double-depleted cells show a significant difference

in PI levels compared to siCtrl after 10 h of treatment (Figure 7B). This confirms our original finding that ESCRTs have a protective role upon endolysosomal membrane damage.

2. The authors conclude that also antihistamine-induced lysosomal damage triggers the recruitment of ESCRT. In order to conclude this, proper quantification and statistics should be applied. Furthermore, it would be important to also address whether ESCRT has a protective effect in the case of antihistamine-induced lysosomal damage.

In the previous manuscript we showed recruitment of ESCRTs to damaged endolysosomal membranes when using different agents (old Supplementary figure 2) such as Astemizole and Terfenadine. The reason for this was a very interesting study from Ellegaard et al. [3] where they identified several cationic amphiphilic drugs as inducers of lysosomal cell death. Therefore we were interested in dissecting early stages of induced lysosomal breakage. Previously, in RPE-1 and HeLa-CHMP4BeGFP-mCherryGAL3 stable cell lines, incubation with 6 μM terfenadine and 15 μM astemizole resulted in mild and sporadic recruitment of ESCRTs. Therefore, we tested higher concentrations of terfenadine (7-8 μM) and astemizole (20 μM up to 40 μM until cells completely collapsed). Upon treatment with terfenadine, HeLa-CHMP4BeGFP-mCherryGAL3 stable cells showed increased lysosomal levels of CHMP4B-eGFP at 2 h as indicated in the EV2B. On the other hand, in the same cell line treatment with 25 μM astemizole showed transient recruitment of ESCRT-III (included Suppl.MovieX4 for reviewers). Interestingly, we observed that the RPE-1 cell line showed more pronounced effects upon treatment with antihistamines and therefore we tested higher concentrations of astemizole and terfenadine than those used for the HeLa cell line. As presented in Fig. EV2B, after 2 h treatment with 7 μM terfenadine, we observed an increase in the number of CHMP4B-eGFP foci per cell when compared to the untreated control. To test whether ESCRT has a protective effect in the case of antihistamine-induced lysosomal damage we transfected RPE-1 cells that stably express CHMP4B-eGFP with control (siCtrl) or siALIX+siTSG101 and performed live-cell imaging (see Suppl.MovieX3 for reviewers). However, cells co-depleted for TSG101 and ALIX showed no obvious increase in cell death after 6 h of terfenadine treatment. This suggests that the type of membrane injury caused by amphiphilic drugs might be distinct from that caused by LLOMe, which presumably requires a different repair process. As we were not able to see robust recruitment using different concentrations of astemizole in both cell lines, we excluded results with this antihistamine from the manuscript.

3. Figures 1, 2, 3, 5 as well as many supplementary figures lack quantification and statistics. Figures 4 and 6 have some asterisks (very small font!) probably indicating significance, but what was compared and how is not indicated in the legend. All conclusions (also negative ones) should be supported by appropriate quantification and statistics!

We agree with the reviewer that it is important to include quantification and statistics, and in all figures (including Expanded view figures) the data have now been properly quantified, with statistical significance indicated in the figure legends. We have also increased font size to improve legibility.

Referee #2:

Minor.

1. The authors indicate that subunits of ESCRT-I /-III but not ESCRT-0 or -II are recruited to damaged lysosomes upon LLOMe treatment (Fig. 2). In these experiments, the cells are treated with LLOMe for 2 h. Could ESCRT-0 or -II be recruited transiently and so missed in these experiments?

We thank the reviewer for this helpful suggestion. We performed a new screen using 250 μm LLOMe and fixed cells after 30 min of treatment instead of 2 h. Interestingly, we were now able to detect EAP30, a component of the ESCRT-II complex, being clearly recruited to damaged endolysosomes (Figure 2). Regarding the ESCRT-0 complex we were not able to see a significant change in the number of foci per cell upon LLOMe treatment when testing endogenous HRS levels (Figure 2) or GFP-HRS (EV3). In addition, upon depletion of HRS we were not able to see any

change in the dynamics of CHMP4B recruitment, indicating that ESCRT-0 does not seem to be involved in the endolysosomal membrane repair (Figure 3).

2. In Fig.4 A, the lysotracker foci are hard to distinguish. Please add high-resolution images.

In the revised manuscript we now present high-resolution images. Yet, as the Lysotracker signal is lost from damaged endolysosomes it is very hard to detect any foci after 10 min and 30 min of LLOMe treatment (see quantification graph).

3. In Fig.7, it seems that combined depletion of TSG101 and ALIX had a similar effect to depletion of TSG101 only, but depletion of ALIX also had some effect on cell death. Please discuss. Show the knockdown efficiency of siRNA oligos in this experiment.

As mentioned in the response to reviewer 1, we have now further investigated the protective role of ESCRTs against endolysosomal membrane damage. Using a new assay as presented in Figure 7, cells depleted of ALIX show a significant difference in Annexin V and propidium iodide positive cells when compared to the siCtrl only after 10 h of treatment with 250 μM LLOMe. Interestingly, after 6 h of treatment siALIX shows significantly lower levels of Annexin V positive cells when compared to siTSG101 indicating a more striking effect of TSG101 depletion on cell viability. The knockdown efficiency of siRNA oligos for this experiment is now included in the new EV5.

References for reviewers:

1. Cummings, B.S. & Schnellmann, R.G. Measurement of cell death in mammalian cells. *Curr Protoc Pharmacol* **Chapter 12**, Unit 12 18 (2004).

2. Aits, S. *et al*. Sensitive detection of lysosomal membrane permeabilization by lysosomal galectin puncta assay. *Autophagy* **11**, 1408-1424 (2015).

3. Ellegaard, A.M. *et al*. Repurposing Cationic Amphiphilic Antihistamines for Cancer Treatment. *EBioMedicine* **9**, 130-139 (2016).

2nd Editorial Decision 31st Aug 2018

Thank you for submitting a revised version of your manuscript. I have looked at both the manuscript and the point-by-point response letter and find that all criticisms have been sufficiently addressed. However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I would kindly ask you to address:

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Harald Stenmark
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99753

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?	The number of experiments was adapted to the expected effect size and the anticipated consistency between the experiments. In general for all presented data at least three independent experiments were performed. The number of individual experiments and the number of cells analyzed are indicated in the figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For confocal microscopy analyses only samples of good staining quality were analyzed.
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.	The experiments presented in the Figures 1,2,5,7 and EV1,2,3 were performed by four different persons. Results presented in the Figure 8 were performed in the lab of our collaborator Professor Frank Lafont.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	Experiments were performed by different persons. Different persons aquired and analysed confocal and super-resolution images and movies.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
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. We tested our datasets for normal distribution and chose an appropriate test accordingly using GraphPad Prism Version 5.01.
Is there an estimate of variation within each group of data?	Standard deviation is used in Figure 1,2, EV1, EV2 and EV3. Standard error of mean is used in Figure 7 and 8.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Rabbit anti-ALIX was used originally in Cabezas A, 2005, J Cell Sci. Rabbit anti-CHMP4B (Sagona et al, 2010, Nat Cell Biol), rabbit anti-HRS (Raiborg et al, 2001,EMBO J), rabbit anti-CHMP3 (Christ et al, 2016, J Cell Biol). Goat anti-GAL3 (AF1154, R&D Systems), anti-human Galectin-3 Alexa Flour 488 (cat. no. IC1154G, R&D Systems), rabbit anti-CHMP2A (10477-1-AP, Proteintech), mouse anti-LAMP1 (H4A3, Developmental Studies Hybridoma Bank), rabbit anti-HD-PTP (10472-1-AP, Proteintech), mouse anti-TSG101 (612697, BD Transduction Laboratories), mouse anti-β-actin (A5316, Sigma-Aldrich). All secondary antibodies used for immunofluorescence studies and western blotting were obtained from Jacksons ImmunoResearch Laboratories or from Molecular Probes (Life Technologies).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa, RPE-1, H460 cell lines were originally obtained from ATCC. HeLa (Kyoto) cells were obtained from D. Gerlich, Institute of Molecular Biotechnology, Wien, Austria. The cell lines are routinely tested for mycoplasma infections every sixth week by the cell lab-manager.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	Analyses scripts will be deposited at https://github.com/koschink/Radulovic_et_al
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).	See point above.
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).	NA
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.	NA

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.	NA