EMBO reports

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Mitochondrial E3 Ligase MARCH5 Regulates FUNDC1 to Fine-tune Hypoxic Mitophagy

Ziheng Chen, Lei Liu, Qi Cheng, Yanjun Li, Hao Wu, Weilin Zhang, Yueying Wang, Sheikh Arslan Sehgal, Sami Siraj, Xiaohui Wang, Jun Wang, Yushan Zhu, and Quan Chen

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Review timeline:

Submission Date:	07 September 2016
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Editorial Decision:	12 December 2016
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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 28 October 2016

Thank you for the submission of your research manuscript to our journal. I apologize for the delay in getting back to you. As one referee was unfortunately not responsive I have made a decision based on two referee reports, which are copied below.

As you will see, both referees acknowledge the potential interest of the findings. However, the referees also point out several seemingly inconsistent observations and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. Both referees suggest more experiments to investigate the interaction between FUNDC1 and MARCH5 in more detail, in order to clarify some unexpected observations. Moreover, both referees suggest quantification of key experiments such as the physical interaction and the induction of mitophagy. The discussion should be extended to present the results and the proposed model in the most appropriate and careful way.

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.

REFEREE REPORTS

Referee #2:

The manuscript by Quan Chen and colleagues examines the regulation of mitochondrial degradation that is mediated by FUNDC1 during hypoxia. They find that the MARCH3E3 ubiquitin ligase is activated by hypoxia to ubiquitinate and degrade FUNDC1. They propose that this regulatory mechanism serves to fine-tune the response of cells to hypoxia. Overall, this is a solid study that clearly identifies MARCH5 is a regulator of FUNDC1 levels. By both overexpression and knockdown studies, they show that MARCH5 can control the level of FUNDC1 and also modulate the level of mitophagy under hypoxia conditions. One important aspect that remains to be clarified is the purpose of this regulatory mechanism. Upon hypoxia, why does MARCH5 initially degrade FUNDC1 but then allows FUNDC1 to persist and degrade mitochondria? This seems counterintuitive and may suggest that the experimental model has some artifactual aspects. This issue is touched upon in the Discussion, but a more detailed discussion would be helpful.

Some other issues that should be addressed:

1) Some of the figures are labeled in a confusing manner. In Fig. 2H, for example, "-shRNA" is confusing because it can imply the lack of shRNA.

2) The authors attribute the region 94-110 in FUNDC1 as being important for physical interaction with MARCH5. However, the experiment in 3E shows substantial interaction in the absence of this region. Because the result is somewhat subtle, it is important to discuss more thoroughly and perform quantification of the physical interaction.

3) Fig. 4D and E would benefit from quantification. The authors say that Fig. 4H shows that K119R is more effective at degrading mitochondria. However, the data is marginal at showing this.

4) In Fig. 4G, it is difficult to conclude that K119R promotes more co-localization between LC3 and mitochondria, based on one image that is complicated to interpret.

5) The authors propose that MARCH5 activity against FUNDC1 is controlled by the oligomeric state of the former. The data supporting this idea is correlative and should be discussed more carefully.

Referee #3:

FUNDC1 serves as a mitophagy receptor under hypoxia. Here, the authors reveal a novel regulatory mechanism of this pathway involving the E3 ligase MARCH5. MARCH5 is shown to ubiquitylate FUNDC1 at K119 triggering its proteasomal degradation. Strikingly, this occurs independently of the E3 ligase parkin that is involved in PINK1-dependent turnover of depolarized mitochondria. Inhibition of FUNDC1 degradation either by mutating K119 or downregulating MARCH5 increases the rate of mitophagy, suggesting that MARCH5-dependent proteolysis fine-tunes mitophagy in hypoxia. Overall, this is an interesting manuscript revealing a novel mode of regulation for mitophagy. Although most experiments were performed under conditions of protein overexpression, the findings are clear-cut and unexpectedly point to distinct roles of E3 ligases in different mitophagy pathways. The authors should, however, consider the following points:

1. The authors demonstrate proteasomal degradation of FUNDC1 but I am uncertain whether or not proteolysis is stimulated in hypoxia. Fig. 2D/E shows degradation of FUNDC1 under normoxic conditions. Does hypoxia accelerate proteolysis? In Fig. 2F/G, the authors observe FUNDC1 proteolysis in MARCH5 knockdown cells while MG132 completely inhibits proteolysis in Fig. 2D/E. Can this be explained by inefficient depletion of MARCH5?

2. In Fig. 4G, the authors describe the formation of LC3 foci upon overexpression of the K119R form of FUNDC1 (but not upon expression of FUNDC1), but this effect is not well documented. The authors should show a quantification of these data to substantiate their conclusion.

3. Related to this point, if the K119R variant of FUNDC1 induces mitophagy, what is the explanation for this? Similar levels of ectopically expressed FUNDC1 and K119R accumulate in these cells according to Fig. 4H.

4. The immunoprecipitation experiment shown in Fig. 6A is puzzling. FUNDC1 levels decrease upon incubation in hypoxia as expected but similar amounts of FUNDC1 are precipitated. Does this indicate that only a small fraction of FUNDC1 (at time point 0) is precipitated under these conditions?

1st Revision - authors' response 02 December 2016

RESPONSE TO THE REVIEWERS

Referee #2:

The manuscript by Quan Chen and colleagues examines the regulation of mitochondrial degradation that is mediated by FUNDC1 during hypoxia. They find that the MARCH3E3 ubiquitin ligase is activated by hypoxia to ubiquitinate and degrade FUNDC1. They propose that this regulatory mechanism serves to fine-tune the response of cells to hypoxia. Overall, this is a solid study that clearly identifies MARCH5 is a regulator of FUNDC1 levels. By both overexpression and knockdown studies, they show that MARCH5 can control the level of FUNDC1 and also modulate the level of mitophagy under hypoxia conditions. One important aspect that remains to be clarified is the purpose of this regulatory mechanism. Upon hypoxia, why does MARCH5 initially degrade FUNDC1 but then allows FUNDC1 to persist and degrade mitochondria? This seems counterintuitive and may suggest that the experimental model has some artifactual aspects. This issue is touched upon in the Discussion, but a more detailed discussion would be helpful.

Response 1: *We thank the referee for the positive assessment of our study. We have discussed more about MARCH5/FUNDC1 axis in the revised manuscript (**Discussion part,** Page10, line17-23). "Unlike Parkin regulated mitophagy that ubiquitylates a large amount of mitochondrial outer membrane proteins and then recruits autophagy adaptor P62 and isolation membrane, MARCH5 fine tunes mitophagy by specifically ubiquitylating and degrading receptor protein FUNDC1 in response to hypoxic stress. Regulation of MARCH5/FUNDC1 axis desensitizes mitochondria and avoids improper clearness to undamaged mitochondria. Our findings uncover the distinct roles of E3 ligases in different mitophagy pathways."*

Some other issues that should be addressed:

1) Some of the figures a re labeled in a confusing manner. In Fig. 2H, for example, "-shRNA" is confusing because it can imply the lack of shRNA.

Response 2: *Many thanks for the suggestions. We have corrected all the improper or informal labels in the revised manuscript.*

2) The authors attribute the region 94-110 in FUNDC1 as being important for physical interaction with MARCH5. However, the experiment in 3E shows substantial interaction in the absence of this region. Because the result is somewhat subtle, it is important to discuss more thoroughly and perform quantification of the physical interaction.

Response 3: *Thanks for the good suggestions. Actually, excessive expression of FUNDC1-Δ94-110-myc truncation plasmid could induce non-specific binding with other proteins. It may cause a small amount of interaction between FUNDC1-Δ94-110-myc truncated variant and GFP-MARCH5 as shown in previous FUNDC1 truncation date. To correct this, full-length and truncated forms of FUNDC1-myc were expressed in similar amounts in HeLa cells expressing GFP-MARCH5.*
As it is evident from the figure below, there was hardly any interaction between GFP-MARCH5 and FUNDC1-Δ94-110-myc truncation.

*But the same was not the case with other truncated forms of FUNDC1-myc (see **Fig 3E in revised manuscript** and **Fig R1**). These results indicate that FUNDC1 indeed interacted with MARCH5 through amino acids 94-110.*

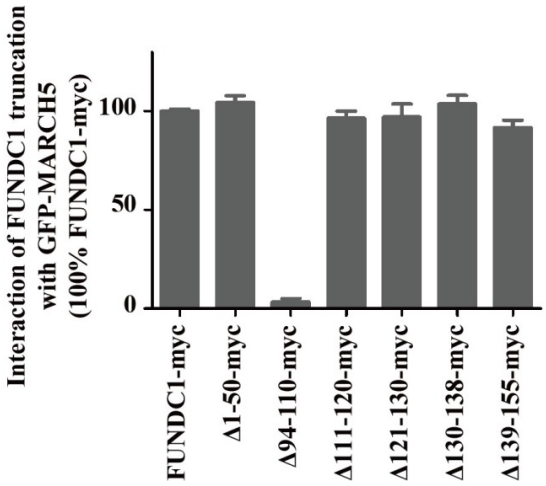

Figure R1. Quantification of interaction between FUNDC1 truncation forms and MARCH5 as indicated in (Fig 3E in revised manuscript) (mean ± SEM, from 3 independent experiments).

3) Fig. 4D and E would benefit from quantification. The authors say that Fig. 4H shows that K119R is more effective at degrading mitochondria. However, the data is marginal at showing this.

***Response 4:** Thanks for your valuable suggestion. The quantification data of Figs 4D-E was shown in **Appendix Figs S2A-B at revised manuscript**, respectively. For Fig 4H, we also quantified the decrease of mitochondrial proteins Tim23 and Tom20 in cells expressing FUNDC1-myc or K119R-myc mutant (**Appendix Fig S3B in revised manuscript**). We believe that these results show that K119R mutant is more effective at degrading mitochondria than wild-type FUNDC1.*

4) In Fig. 4G, it is difficult to conclude that K119R promotes more co-localization between LC3 and mitochondria, based on one image that is complicated to interpret.

***Response 5:** We are grateful for your insight that helped us strengthen our data. In order to accurately detect the effect of K119R mutation on FUNDC1 mediated mitophagy, we quantified the GFP-LC3 aggregates surrounding mitochondria in FUNDC1-myc or K119R-myc positive cells treated as in (Figure 4G) with ImageJ (mean ± SEM; n = 200 cells from three independent experiments) (please see **Appendix Fig S3A at revised manuscript**). The statistic analysis showed that K119R promoted more co-localization between LC3 and mitochondria than wild-type FUNDC1.*

5) The authors propose that MARCH5 activity against FUNDC1 is controlled by the oligomeric state of the former. The data supporting this idea is correlative and should be discussed more carefully.

***Response 6:** Thanks for your suggestion. We added the sentences at revised manuscript (Page9, line5-7) to make the conclusion more accurate. "We believe that the oligomeric switch of MARCH5 is important for degradation of FUNDC1, however exact nature of this complex (oligomeric) and its interaction needs to be explored further."*

Referee #3:

FUNDC1 serves as a mitophagy receptor under hypoxia. Here, the authors reveal a novel regulatory mechanism of this pathway involving the E3 ligase MARCH5. MARCH5 is shown to ubiquitylate FUNDC1 at K119 triggering its proteasomal degradation. Strikingly, this occurs independently of the E3 ligase parkin that is involved in PINK1-dependent turnover of depolarized mitochondria.

Inhibition of FUNDC1 degradation either by mutating K119 or downregulating MARCH5 increases the rate of mitophagy, suggesting that MARCH5-dependent proteolysis fine-tunes mitophagy in hypoxia. Overall, this is an interesting manuscript revealing a novel mode of regulation for mitophagy. Although most experiments were performed under conditions of protein overexpression, the findings are clear-cut and unexpectedly point to distinct roles of E3 ligases in different mitophagy pathways. The authors should, however, consider the following points:

1. The authors demonstrate proteasomal degradation of FUNDC1 but I am uncertain whether or not proteolysis is stimulated in hypoxia. Fig. 2D/E shows degradation of FUNDC1 under normoxic conditions. Does hypoxia accelerate proteolysis?

Response 7: *We're thankful to you for picking up on this. To explore the FUNDC1 proteolysis under hypoxia, we exposed cells in 1% O_2 for the indicated time, with or without MG132 (10 μM), and detected the FUNDC1 protein level by western blotting analysis (**Figs R2A-B**). Consistent with the results in Fig 1C at revised manuscript, FUNDC1 proteolysis was indeed accelerated under hypoxic condition, and blocked by MG132, a specific proteasome inhibitor.*

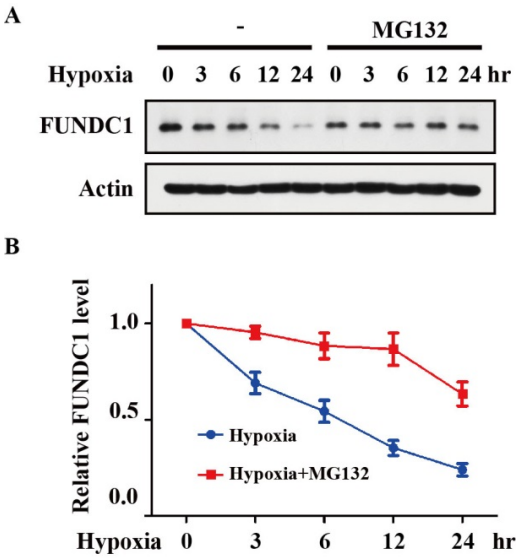

Figure R2. (A) HeLa cells were exposed to 1% O_2 for the indicated time, with or without MG132 (10 μM), and then the FUNDC1 protein was detected by western blotting. (B) Quantification of FUNDC1 level as indicated in (A) (mean ± SEM, from 3 independent experiments).

In Fig. 2F/G, the authors observe FUNDC1 proteolysis in MARCH5 knockdown cells while MG132 completely inhibits proteolysis in Fig. 2D/E. Can this be explained by inefficient depletion of MARCH5?

Response 8: *Wild type and mutant MARCH5 were expressed with HeLa cells instead of MARCH5 knockdown cells in Figs 2F-G, as mentioned in our primary manuscript (page6, line18). We made a typographical error in Figure legends part of Fig 2F (primary manuscript, page 13, line 19). Now we have corrected it in revised manuscript (page13, line19 at revised manuscript) and we apologize for that.*

2. In Fig. 4G, the authors describe the formation of LC3 foci upon overexpression of the K119R form of FUNDC1 (but not upon expression of FUNDC1), but this effect is not well documented. The authors should show a quantification of these data to substantiate their conclusion.

Response 9: *Thanks for suggestion. We quantified the GFP-LC3 aggregates surrounding mitochondria in FUNDC1-myc or K119R-myc positive cells (mean ± SEM; n = 200 cells from three independent experiments) (please see **Appendix Fig S3A at revised manuscript**). The statistical analysis showed that K119R promoted more co-localization between LC3 and mitochondria than wild-type FUNDC1.*

3. Related to this point, if the K119R variant of FUNDC1 induces mitophagy, what is the explanation for this? Similar levels of ectopically expressed FUNDC1 and K119R accumulate in these cells according to Fig. 4H.

Response 10: *Thanks for great suggestion. For Fig 4H, we quantified the decrease of mitochondrial proteins Tim23 and Tom20 in cells expressing FUNDC1-myc or K119R-myc mutant, and found K119R mutant was more effective at degrading mitochondria than wild-type FUNDC1 (**Appendix Fig S3B in revised manuscript**). A likely conclusion could be that K119R mutant increases the protein stability of FUNDC1 and makes it easier to recruit LC3 and induce mitophagy.*

4. The immunoprecipitation experiment shown in Fig. 6A is puzzling. FUNDC1 levels decrease upon incubation in hypoxia as expected but similar amounts of FUNDC1 are precipitated. Does this indicate that only a small fraction of FUNDC1 (at time point 0) is precipitated under these conditions?

Response 11: *Thanks for your kind concern. To quantify the immunoprecipitated FUNDC1 in Fig 6A, grayscale values of the precipitated FUNDC1 bands, measured with ImageJ software, are shown under the corresponding bands to indicate the band intensities (please see **Fig 6A in revised manuscript**). Immunoprecipitated FUNDC1 was indeed somewhat decreased in a time-dependent manner in cells exposed to hypoxia because of reduction of total FUNDC1 protein. Under these conditions, immunoprecipitated MARCH5-myc was significantly increased, supporting the conclusion that hypoxia progressively enhanced the interaction between MARCH5 and FUNDC1.*

2nd Editorial Decision 12 December 2016

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below. As you will see, both referees are very positive about the study and request only a minor change to the text. From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

REFEREE REPORTS

Referee #2:
The revised manuscript is improved and has addressed my concerns. In the quoted passage in response 1, the wording is awkward, and I would suggest the following: "Regulation of the MARCH5/FUNDC1 axis desensitizes mitochondrial degradation and avoids improper clearance of undamaged mitochondria."

Referee #3:
The authors have carefully addressed my concerns when preparing the revised manuscript. I feel that the identification of a novel ubiquitin-dependent mechansims fine-tuning mitophagy will be of broad interest in the field.

2nd Revision - authors' response 14 December 2016

The authors made the requested changes and submitted the final version of the manuscript.

3rd Editorial Decision 20 December 2016

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

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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:	Quan Chen
Journal Submitted to:	EMBO Reports
Manuscript Number:	2016-43309V2

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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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?	Based on the previous studies. For the cell imaging experiments, n>100.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N\
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion except for the bad imaging quality.
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 cells were randomly selected for imaging and analyzing.
For animal studies, include a statement about randomization even if no randomization was used.	N\
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.	Yes.We used double-blind method for some cell imaging observation and statistic analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N\
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
Is there an estimate of variation within each group of data?	No
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).	SDS-PAGE and western blotting section, Materials and Methods Part.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N\
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\
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N\

14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N.I.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N.I.
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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.	N.I.

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.I.
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.I.
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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.I.

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.I.