EMBO Molecular Medicine

IGSF10 mutations dysregulate gonadotropin-releasing hormone neuronal migration resulting in delayed puberty

Sasha R Howard, Leonardo Guasti, Gerard Ruiz-Babot, Alessandra Mancini, Alessia David, Helen L Storr, Lousie A Metherell, Michael JE Sternberg, Claudia P Cabrera, Helen R Warren, Michael R Barnes, Richard Quinton, Nicolas de Roux, Jacques Young, Anne Guiochon-Mantel, Karoliina Wehkalampi, Valentina Andrè, Yoav Gothilf, Anna Cariboni, Leo Dunkel

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Transfer date:	26 January 2016
Editorial Decision:	12 February 2016
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Accepted:	14 March 2016

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.)

Editor: Céline Carret

1st Editorial Decision 12 February 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees who we asked to evaluate your manuscript.

You will see that both referees are enthusiastic about the study and only request minimal revision work. I will be happy to invite a revision of your manuscript if you can address the issues that have been raised within 3 months. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

In order to gain time, shall the manuscript be later accepted, I would like to suggest taking care of the below editorial requirements at the same time.

I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The study presents novel findings that will help in the diagnosis of delayed puberty in patients and will contribute to our understanding of the mechanisms governing GnRH migration and development.

Referee #1 (Remarks):

In the present study, Howard and colleagues present a compelling series of human genetic, in vitro and in vivo studies that elegantly describe the novel role of IGSF10 in the migration of GnRH neurons to the hypothalamus during the embryonic period. Their data is supported by a large analysis of patients suffering hypogonadotropic hypogonadism and the function of this molecule postulated and assessed by in vitro models using GN11 cells, which showed reduced migration after Igsf10 knockdown, and reduced migration of GnRH neurons in zebra fish with a morpholino knockdown approach of this molecule. Overall, the study is innovative, informative, well designed and the results clearly stated. There are only a few comments regarding the proposed mechanism of action for Igsf10:

- The authors tested the migration of GN11 cells after KD of Igsf10. This experiment assumes that GnRH neurons express Igsf10, which would be acting, perhaps, in an auto synaptic feedback loop in GnRH neurons. Still, the authors showed the expression of Igsf10 in other hypothalamic areas and, in the discussion, mentioned that this molecule probably participates in the creation of a gradient needed to direct GnRH neuronal migration. It is therefore not clear whether GnRH neurons may also express this molecule or whether GN11 cells, due to their immortalized nature, are not a faithful replication of GnRH neurons in vivo. It would be good if the authors clarified this by assessing the expression of Igsf10 in other GnRH cell lines and, if possible, through double label ISH with better resolution than the images presented in Figure 4.

- The authors nicely explain that the amount of mutations accumulated in a single individual may account for the different magnitudes in the HH phenotype observed, however, this does not explain the adult onset of HH discussed inlines 321+. If the role of Igsf10 is solely in the migration of GnRH neurons, as suggested by the disappearance of its expression in late embryonic phases, why would these mutations induce secondary amenorrhea after the HPG axis has been properly activated during puberty? Do they know whether this molecule has further developmental regulation? Would it be possible that its expression increases again at the time of puberty onset and/or is regulated by sex steroids in adulthood? Including a few samples from mice at critical developmental time points (e.g. infantile, juveline, peripubertal and adult) would address this question.

- Line 193: do they mean "presence" instead of absence?
- Line 197: One of the mutations has less than 3 D and would be therefore "possibly damaging" according to their criteria.
- Figure 4: The data depicting IGSF10 expression in the human tissue is too weak. Are they sure this is specific? Please, include controls using the sense probe in the supplementals.
- Figure 4: Please, include a scale bar in each panel.

Referee #2 (Comments on Novelty/Model System):

The authors demonstrate that IGSF10 regulates embrionic GnRH neuronal migration and mutations result in delayed puberty. This is, indeed, a new concept in Molecular Medicine. The manuscript has a high technical quality and the information is novel.

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puberty in humans with common genetic basis for functional hypogonadotropic hypogonadism. Indeed, this is the first time that this has been demonstrated as a casual mechanism in delayed puberty.

This is a beautiful manuscript with important data to better understand patients with delayed puberty and hypogonatotropic hypogonadism. With the study, very elegant methodology was used. It is well written and easy to read.

Comments:

1. The Introduction should be shortened. Background on IGSF10 should be included in this section.

2. After whole exome sequencing and targeted exome sequencing, the authors found 4 mutations (all of them are heterozygous missense variants predicted to be deleterious by {greater than or equal to}3/5 prediction tools) in IGSF10 (3 of them present in public databases). This is important information; however, with what certitude are these variants pathological?

3. To your knowledge, what kind of differences can be established between mutations in IGSF10 and IGSF1 genes in relation with delayed puberty?

4. Do the authors see any differences in the phenotype between patients with IGSF10 mutations and patients with mutations in KAL1 or PROKR2?

5. In table III the characteristics of Delayed Puberty Probands indicate that the sex is predominantly males (9 out of 10). Any specific comment about the only female subject? Regarding estradiol levels in males, did you measure them?

6. It looks like the induction of puberty was done only in 5 patients. Could the authors comment on the response and the degree of puberty obtained?

7. If would be of interest to include in Table IV data regarding the final height in the patients if you have it.

1st Revision - authors' response 01 March 2016

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The in vitro migration assay shown in the study involves shRNA knockdown of Igsf10 in NIH3T3 cells, a mouse fibroblast derived cell line, which we have shown to have high endogenous expression of Igsf10. The evidence from our in situ hybridization studies of Igsf10 expression in the nasal mesenchyme led to the hypothesis that Igsf10 acts as a chemokine to influence GnRH neuronal migration. Thus we did not anticipate that GnRH neurons or GN11 cells would express Igsf10 and did not attempt to knockdown Igsf10 in GN11 cells. Instead, we used NIH3T3 cells as a model of nasal mesenchyme tissue to demonstrate that knockdown of Igsf10 in these cells leads to reduced migration of the GN11 cells plated alongside, in comparison to those plated alongside NIH3T3 cells with normal Igsf10 expression. The manuscript has been modified to clarify this experimental set-up –

Lines 267-272: 'We performed co-culture experiments of GN11 aggregates placed on confluent NIH3T3 monolayers. NIH3T3 cells, derived from a mouse embryonic fibroblast cell-line, express high levels of endogenous Igsf10. The NIH3T3 cells were treated with scrambled- or Igsf10-shRNAs, the latter leading to highly reduced levels of Igsf10 expression (Fig 5A).'

- The authors nicely explain that the amount of mutations accumulated in a single individual may account for the different magnitudes in the HH phenotype observed, however, this does not explain the adult onset of HH discussed inlines 321+. If the role of Igsf10 is solely in the migration of GnRH neurons, as suggested by the disappearance of its expression in late embryonic phases, why would these mutations induce secondary amenorrhea after the HPG axis has been properly activated during puberty? Do they know whether this molecule has further developmental regulation? Would it be possible that its expression increases again at the time of puberty onset and/or is regulated by sex steroids in adulthood? Including a few samples from mice at critical developmental time points (e.g. infantile, juveline, peripubertal and adult) would address this question.

An overlapping phenotype between DP and HA has been seen in a previous study (Caronia et al, NEJM 2011, DOI: doi: 10.1056/NEJMoa0911064), referred to in our manuscript in lines 408-409. The same authors have proposed the mechanism that reduced GnRH neuronal numbers (caused by mutations in e.g. KAL-1 or PROKR2) may lead to both HH and HA. We hypothesise that mutations in IGSF10 may also lead to reduced numbers and/or late arrival of GnRH neurons at the hypothalamus during embryonic life, resulting in defective functionality of the GnRH neuroendocrine network. This mechanism may therefore lead to either delayed onset of puberty or reduced capacity of the HPG axis to respond in times of compromise e.g. in excessive exercise, reduced caloric intake or other stressors that cause HA.
Unfortunately we do not have definitive data on the timing of puberty of our HA patients to discover whether they also had DP, but the study by Caronia et al reports DP in 25% of patients with HA. Interestingly, some partial forms of IHH may lead to normal timing of puberty but arrested puberty or infertility later in life, also suggesting that defects in GnRH neuronal function may present after puberty onset. Additionally, we have previously carried out expression studies using in situ hybridisation in peri-pubertal mice and did not see any expression of Igsf10 in peri-pubertal mouse hypothalamus.
The manuscript discussion has been modified to give further detail in response to this point –

Lines 412-416: 'Specifically, this clinical variability can result from mutations in genes such as KAL1, PROKR2 and now IGSF10, which may lead to late arrival or reduced numbers of GnRH neurons to the hypothalamus, thus compromising the function of the GnRH network.'

- Line 193: do they mean "presence" instead of absence?
Filtering of variants based on the exclusion of those found in public databases is a frequently used step in the filtering pipeline in rare diseases. However, in a more common condition such as delayed puberty this filtering step is not appropriate, as we expect to find 'disease-causing' mutations in the public databases, as we find individuals with delayed puberty in the public databases. Thus it is the 'absence' of these variants that cannot be used as one of the exclusion criteria.

- Line 197: One of the mutations has less than 3 D and would be therefore "possibly damaging" according to their criteria.
Damaging or possibly damaging were both assessed as 'deleterious' by our pipeline, but this clarification has been included in the revised manuscript –

Line 184-6: 'All four IGSF10 variants are heterozygous missense variants predicted to be deleterious, damaging or possibly damaging by ≥3/5 prediction tools (Table 2).'

- Figure 4: The data depicting IGSF10 expression in the human tissue is too weak. Are they sure this is specific? Please, include controls using the sense probe in the supplementals.
Panel N is the expression image for the sense probe for human IGSF10, which shows no visible expression, as compared to the purple-colour nasal mesenchyme staining for IGSF10 seen in panels K, L and M.

- Figure 4: Please, include a scale bar in each panel.
Included in revised manuscript.

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Sasha et al, with the corresponding author being Prof. Dunkel, present an elegant multinational study where they have identified mutations in IGSF10 in 6 unrelated families, resulting in intracellular retention of this protein, thus with failure in the secretion of mutant proteins. Furthermore, the authors show that knock out of IGSF10 caused reduced migration of immature GnRH neurons in vitro and perturbed migration and extension of GnRH neurons in a gnrh3:EGFP zebrafish model. Furthermore, loss-of-function mutations in IGSF10 were identified in patients with hypothalamic amenorrhea. The authors conclude by saying that mutations in IGSF10 cause delayed puberty in humans with common genetic basis for functional hypogonadotropic hypogonadism. Indeed, this is the first time that this has been demonstrated as a casual mechanism in delayed puberty.
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Comments:

1. The Introduction should be shortened. Background on IGSF10 should be included in this section.

Please find the introduction shortened as requested. We do believe, however, that discussion of IGSF10 should not appear in the introduction, as this is the main discovery of the study and so details of this gene would logically follow in the results section. IGSF10 was not a candidate gene prior to the start of the study, and the study design was based on an unbiased analysis of WES data, which makes it difficult to highlight one gene in the introduction.

We feel that earlier disclosure would preempt this exciting discovery in the results section, and interrupt the flow of the argument. We are happy to take further guidance from the editor on this point.

2. After whole exome sequencing and targeted exome sequencing, the authors found 4 mutations (all of them are heterozygous missense variants predicted to be deleterious by {greater than or equal to}3/5 prediction tools) in IGSF10 (3 of them present in public databases). This is important information; however, with what certitude are these variants pathological?

Our in vitro data demonstrates failure of secretion of the two N-terminal mutations and retention within the intracellular compartment, which we believe shows clear evidence of their pathogenicity. These mutations were found in 6 out of the 10 families identified. It has not been possible to reproduce these studies for the two C-terminal mutations despite many months of trying to express the full-length and C-terminal protein in mammalian cells. Thus, although our in silico predictions give evidence for predicted pathogenicity of the two C-terminal variants, we are not able to conclusively show these to be pathogenic, as we declare in lines 339-340: 'We have identified an additional two rare variants of unknown significance in 4 further families.'

3. To your knowledge, what kind of differences can be established between mutations in IGSF10 and IGSF1 genes in relation with delayed puberty?

None of our patients with IGSF10 mutations had thyroid abnormalities or other features of the IGSF1 syndrome, apart from delayed onset of puberty. One previous publication describes the sequencing of IGSF1 in families from our DP cohort – Joustra et al, Eur J Pediatr 2015 (DOI 10.1007/s00431-014-2445-9). No pathogenic variants in IGSF1 were found in our cohort with 'simple' delayed puberty, again suggesting that mutations in IGSF1 do not cause self-limited delayed puberty in the absence of other features of the IGSF1 syndrome.

4. Do the authors see any differences in the phenotype between patients with IGSF10 mutations and patients with mutations in KAL1 or PROKR2?

All three of these genes are important in early development and specifically for the correct migration of GnRH neurons to the hypothalamus during embryonic life. Mutations in all three are seen in families segregating with HA, DP, and in the case of KAL1 and PROKR2, IHH or KS. However, we have not conclusively demonstrated as yet that mutations in IGSF10 alone lead to IHH or KS. Please see the addition to the discussion in the main text to further emphasise this point (lines 412-416): 'Specifically, this clinical variability can result from mutations in genes such as KAL1, PROKR2 and now IGSF10, which may lead to late arrival or reduced numbers of GnRH neurons to the hypothalamus, thus compromising the function of the GnRH network.'

5. In table III the characteristics of Delayed Puberty Probands indicate that the sex is predominantly males (9 out of 10). Any specific comment about the only female subject? Regarding estradiol levels in males, did you measure them?

We have previously demonstrated that although there is referral bias in the probands seen in the clinic, exploration of their extended families demonstrates a near equal gender distribution i.e. 1.2males:1female (Reference: Wehkalampi et al, J Clin Endocrinol Metab 2008, DOI 10.1210/jc.2007-1786). Our finding of 9 of 10 of the probands with IGSF10 mutations, but a total of 8 male relatives with DP and 6 female with DP with pathogenic IGSF10 mutations, is consistent with this. We do not believe that IGSF10 mutations are more commonly associated with males and there were no specific phenotypic attributes in the one female proband identified.
Estradiol was not routinely measured in male patients.

6. It looks like the induction of puberty was done only in 5 patients. Could the authors comment on the response and the degree of puberty obtained?

In our large DP cohort, approximately 50% of patients chose induction. As part of the protocol for diagnosis of self-limited DP, all patients were followed up off treatment until full development (Tanner G4+) was achieved. The manuscript has been amended to add this clarification (lines 447-

450): 'In the 50% of patients from the cohort who choose to have pubertal induction via the use of exogenous sex steroids, all patients were followed up once off treatment until the point of full pubertal development (Tanner stage G4+ or B4+) to ensure pubertal development did not arrest off treatment.'

7. If would be of interest to include in Table IV data regarding the final height in the patients if you have it.

This has now been included as an extra column in Table IV, and in the main text lines 207-208: 'At adult height, all but 2 probands (3.III.2 and 6.II.1) fell within normal limits for distance to target height (Table 4) (Saari et al, JAMA Pediatr 2015 DOI: 10.1001/jamapediatrics.2015.25).'

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: Professor Leo Dunkel
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2016-06250-T

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).

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?	Line 713-723
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Line 713-723
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Line 717-718
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	It was not possible to utilise randomisation methods for the zebrafish experiments, due to the nature and conditions of the experiments.
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.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not feasible due to the nature of effects of the Igsf10 morpholinos i.e. it was possible to identify those embryos that had received Igsf10 MO injections by their phenotype.
5. For every figure, are statistical tests justified as appropriate?	Yes - Line 766-776
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes - data symmetrically distributed around the mean on distribution plotting; Shapiro-Wilk test of normality used in SPSS
Is there an estimate of variation within each group of data?	Yes - Line 766-776
Is the variance similar between the groups that are being statistically compared?	Yes - s.e.m. Fig 3C and Fig 5A, D and G

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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).	Line 606-609, line 632-635
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Line 591-594, line 646-651

* 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.	Line 614-616, line 687-690
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mice work - line 760-762; Zebrafish work - zebrafish embryo experiments up until day 5 post fertilisation do not require home office licencing
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.	Yes - as above, and including line 760-762

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Line 754-764
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.	Line 754-764
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Ethical permission not granted to publish human data in public databases
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
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/A

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	The ethical consents gained from the study participants did not include permission to publish genetic data in public databases. Additionally, this whole exome sequencing data is the source of ongoing gene discovery projects that are central to the corresponding author's ongoing research portfolio. As such, although amendments to the original ethical permissions are being sought, this data cannot yet be deposited in a public repository.
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
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).	See above (18)
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
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.	In silico analysis provided in full in EV and Appendix files

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