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Codon identity regulates mRNA stability and translation efficiency during the maternal-to-zygotic transition

Ariel A. Bazzini, Florencia del Viso, Miguel A. Moreno-Mateos, Timothy G. Johnstone, Charles E. Vejnar, Yidan Qin, Dr. Jun Yao, Mustafa K. Khokha and Antonio J. Giraldez

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1st Editorial Decision 02 June 2016

Thank you again for submitting your manuscript along with referee reports and your point-by-point response for consideration by the EMBO Journal. It has now been seen by an arbitrating advisor whose comments are shown below. I also contacted another advisor who added a few more suggestions for the analysis of codon vs amino acid effects that I would suggest you to incorporate in the manuscript. In my view, this re-emphasises what we discussed before: that there is an effect for both and that this is an important and interesting part of the manuscript.

As you will see below our advisor is overall positive about your findings and the response to the referee comments from the previous round, and consequently this person supports publication of your manuscript in The EMBO Journal. The advisor does ask for additional discussions/clarifications on a few points and I would therefore ask you to address this in a final revised version.

Thank you again for giving us the opportunity to consider your work for publication. I look forward to your revision. In addition, feel free to contact me with any questions on the remaining minor revisions asked by the advisors.

Arbitrating advisor:

The manuscript by Bazzini et al. shows a comparative analysis of codon identity/translation impact in mRNA stability, with an emphasis in vertebrate MZT. This manuscript has already gone through a revision process and, in its current version; the amount of work/information included is impressive and the quality of the data/experiments is impeccable. The subject of the work, codon optimality in gene expression regulation, has recently awaken great (re)interest with several key studies in bacteria and yeast and one (published while this manuscript was under revision) in zebrafish. The conclusion of these manuscripts was that codon optimality influences mRNA translation and stability. In the broad sense this is also the main conclusion of this work. Published studies are, to a certain extent, descriptive/correlative with very limited insight into the subjacent mechanism, this limitation is shared by Bazzini's reflecting the current status of the field.

This work contains some specificity over published studies, focusing in MZT and comparing vertebrates (Mainly Xenopus and Zebrafish and to some extent mouse) with an invertebrate (Drosophila) and externally vs. internally developing animals. The conclusion is that the codon optimality effect seems to be conserved along evolution (also with bacteria and yeast). Although it is not completely clear it seems that it could be concluded from this work that it is conserved from MZT to somatic tissues. Thus there is no particular implication for maternal mRNAs degradation after MZT (if that is not the case it would need to be better explained in the manuscript).
Thus, as noted in previous reviews, the conclusions of this work are mainly confirmative. However, I would argue that, in the current status of the field, the differences in how the data are obtained (i.e. the screening in Fig 1) and analyzed, the comparative value of the different species and developmental stages (Conservation through evolution and development is a relevant conclusion) and the specifics of some of the validation experiments make this work of interest and, in my opinion, justify publication.

From the specific points by previous reviewers, I think that most of the criticisms are addressed in the current version of the manuscript to a reasonable extent with the following exception:
1- Reviewer #1 point 2: The effect of CHX on the miRNA regulated mRNAs stability is not obvious, it needs a more detailed explanation.

In addition,
Fig 5:
1- Recent work by Narry Kim's group shows that Oligo dT specifically discriminates below (aprox) 30 nts but not above. Thus pA/R0 does not measure the extent of polyadenylation in a linear manner but with a threshold at aprox 30 nts (i.e. it will differentiate from 25 to 50 but not from 100 to 200). Of relevance to this work, this short range is close to the length of the stored maternal mRNAs. This should be commented and included in the interpretation of the analyses.
2- Poly(A) tail length affects translation (at least the switch from short 25 to long 80, probably not above a certain threshold) and more for non-efficient mRNAs (Long 5' UTRs) than for efficient mRNAs (HS or globin mRNA) but also translation globally affects poly (A) tai length (as shown by CHX treatment). Thus data from fig 5 can be interpreted as both cause and consequence and this should be discussed.
3- As presented, it is difficult to extract quantitative conclusions from fig 5E (even a simple western-blot will be more quantitative).
I'm not sure how much this experiment contributes to the main conclusions of the work (see point 3 from reviewer #1 and the response from the authors). I seems more logical to leave this for future studies, but if the authors want to maintain this experiment quantification should be improved.

Fig 3.
It seems that the effect of non-optimal codons in mouse (distinct from X, ZF and DM in that it has internal development, with a much smaller pool of maternal mRNAs and MZT at the two cell stage) is much reduced compared with X, ZF and DM. This may be relevant and should be commented.

Additional input/suggestion from other advisor contacted on the relative contribution from codon vs amino acid identity:

'There is some codon effect (Fig 1D) as for some amino acids, there appear to be good and bad codons (though the effects are modest when compared to Presnyak et al. in terms of R value).

However, for many amino acids, either all codons are good or all codons are bad - this can be most simply extracted by visual analysis of the same Figure 1D. If all codons are good, then this suggests that the amino acid is relevant, not the codon. The analysis in Figs 4B and 4C is confusing to me but in principle expresses this idea. If you look at where codons cluster in Figures 4B and C it is clear that the majority of codons encoding a given amino acid have similar net effects on CSC (i.e. Leucine is always mildly destabilizing at best or largely destabilizing at worst). Furthermore, looking at Figure 4D, we find the correlation with amino acid identity to be even greater in range than codon identity. We think it would be better and more simply discussed by colouring the codons by amino acid in Fig 1D. Also, the amino acid effect seems stronger and thus would be better dealt with more carefully and throughout the text. In conclusion there seems to be evidence of both effects; however, the amino acid effects are stronger and more interesting. They're certainly deserving of more discussion than the two paragraphs devoted on page nine of the manuscript. I think if the authors were more willing to be open to these ideas, they would write a clearer manuscript that better summarizes their data, rather than forcing it to look like Presnyak et al.'

1st Revision - authors' response 10 June 2016

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This work contains some specificity over published studies, focusing in MZT and comparing vertebrates (Mainly Xenopus and Zebrafish and to some extent mouse) with an invertebrate (Drosophila) and externally vs. internally developing animals. The conclusion is that the codon optimality effect seems to be conserved along evolution (also with bacteria and yeast). Although it is not completely clear it seems that it could be concluded from this work that it is conserved from MZT to somatic tissues. Thus there is no particular implication for maternal mRNAs degradation after MZT (if that is not the case it would need to be better explained in the manuscript).

Authors: While this mechanism is present in other developmental and homeostatic contexts (similar to microRNAs, RNA binding proteins and RNA modifications) codon optimality clearly explains part of the differential mRNA stability in the maternal to zygotic transition. However, we still do not know it role in other transitions. Based on the point raised by the reviewer we have added to following text:

...they are under the regulation of codon optimality. ***The codon optimality code defined during early embryogenesis (Fig 1) correlates with codon bias in homeostasis (Fig 4), suggesting that the code is conserved between early embryogenesis (MZT) and somatic tissues. However, further experiments defining codon optimality code in specific cell types or under different physiological conditions i.e. stress; will define how the code changes with cell identity and cellular state.*** *In addition to tRNA levels,.....*

Thus, as noted in previous reviews, the conclusions of this work are mainly confirmative. However, I would argue that, in the current status of the field, the differences in how the data are obtained (i.e.

the screening in Fig 1) and analyzed, the comparative value of the different species and developmental stages (Conservation through evolution and development is a relevant conclusion) and the specifics of some of the validation experiments make this work of interest and, in my opinion, justify publication.

From the specific points by previous reviewers, I think that most of the criticisms are addressed in the current version of the manuscript to a reasonable extent with the following exception:
1- Reviewer #1 point 2: The effect of CHX on the miRNA regulated mRNAs stability is not obvious, it needs a more detailed explanation.

Authors: We observe that blocking translation with CHX also affect microRNA mediated decay and to a lesser extent deadenylation. We still don't fully understand the mechanism by which miR-430 mediated regulation is also affected by blocking translation. We know that in the presence of CHX, miR-430 is correctly expressed and processed; miR-430 targets get deadenylated (Poly-A fold change 6h-2h+CHX) but the mRNA decay is compromised (Total RNA fold change 6h-2h+CHX), suggesting that the mRNA decay machinery might be affected. In the case of codon optimality both decay and deadenylation are affected in the presence of CHX, showing a difference between the microRNA pathway and codon optimality. While we cannot rule out the possibility that the decay machinery is affected, we can conclude that cycloheximide affects codon optimality. In addition, we show four independent evidences that the codon optimality depends on translation:
1- The reporter libraries (rCSI) compare more than a million different ORFs in the absent or presence of translation (with or without MO) in zebrafish and Xenopus (Figure 1.c).

2- We only see differences in the mRNA decay of endogenous genes enriched in optimal codon compared to the ones enriched in non-optimal when we analyzed the codon composition in the correct frame. We do not see mRNA decay differences when we analyzed genes enriched in optimal or non-optimal 1 or 2 nucleotides out of frame, indicating that the effect is derived from the codon and not from a sequence motif (Figure 1h and supplementary figure 2).

3- Injection of pairs of mRNA that only differed in 1 nucleotide insertion but have opposite codon optimalities, resulting in very different rate of decay according to their optimality (Figure 1i and supplementary figure 2).

4- Injection of specific of morpholinos affects endogenous mRNA decay (Figure 2.c).

We hope the reviewer understand our point that the decay of microRNA targets is part of another study led by other lab members and it is out of the scope of the codon optimality work.

In addition,
Fig 5:
1- Recent work by Narry Kim's group shows that Oligo dT specifically discriminates below (aprox) 30 nts but not above. Thus pA/R0 does not measure the extent of polyadenylation in a linear manner but with a threshold at aprox 30 nts (i.e. it will differentiate from 25 to 50 but not from 100 to 200). Of relevance to this work, this short range is close to the length of the stored maternal mRNAs. This should be commented and included in the interpretation of the analyses.

Authors: We agree with the reviewer's comments. Now, we wrote that "this provides an indirect measurement of the polyadenylation status, ***and represents the proportion of deadenylated and polyadenylated mRNAs rather than an direct measurement of their length (Bazzini, Lee et al. 2012, Park, Yi et al. 2016)****".*

2- Poly(A) tail length affects translation (at least the switch from short 25 to long 80, probably not above a certain threshold) and more for non-efficient mRNAs (Long 5' UTRs) than for efficient mRNAs (HS or globin mRNA) but also translation globally affects poly (A) tai length (as shown by CHX treatment). Thus data from fig 5 can be interpreted as both cause and consequence and this should be discussed.

Authors: The reviewer is absolutely right. While we don't state whether the poly-A length is affecting translation or whether translation is affecting poly-A length, the field will read this as cause of the reduction of translation. Thus we have added the following sentences in the discussion.

....influences the level of translation (Fig 5). ***However, we cannot distinguish whether the poly-A length changes are the cause or consequence of the translation differences caused by codon optimality. In addition,*** *we cannot exclude that additional features....*

3- As presented, it is difficult to extract quantitative conclusions from fig 5E (even a simple western-blot will be more quantitative).
I'm not sure how much this experiment contributes to the main conclusions of the work (see point 3 from reviewer #1 and the response from the authors). I seems more logical to leave this for future studies, but if the authors want to maintain this experiment quantification should be improved.

Authors: Based on the reviewer's comment we have quantified the GFP levels compared to DsRed and added it in the figure 5E.

Fig 3.
It seems that the effect of non-optimal codons in mouse (distinct from X, ZF and DM in that it has internal development, with a much smaller pool of maternal mRNAs and MZT at the two cell stage) is much reduced compared with X, ZF and DM. This may be relevant and should be commented.

Authors: We appreciated the reviewer comments. We agree that the codon optimality effect during the maternal to zygotic transition in mouse shown in figure 3 is smaller than the rest, however it is important to consider that this estimates are based on conservation of the codon optimality code, and might be derived from different mechanisms of regulation or from differences in part of the code. As the reviewer points out, the timing and number of cell divisions in mouse is quite different from the other species analyzed. Therefore, we prefer not to speculate whether codon optimality is "stronger" in one species that in the other one because it would require extensive experimental and computational analysis to support those claims. For example, we should consider more time points, we should define the codon optimality code in mouse, similar to the analysis that we have performed in Figure 1 for zebrafish and Xenopus. However, we agree that this is a interesting observation and therefore we modified the text:

We observed that optimal codons were depleted and non-optimal codons were enriched in unstable mRNAs, consistent with a conserved role of codon optimality in the regulation of mRNA stability during the MZT across animals (Fig 3G-J) (P= 6.8e-12 zebrafish, 5e-11 Xenopus, 1e-03 mouse, 3e-04 Drosophila, Spearman). ***While the effect of non-optimal codons appears to be weaker in mouse that in other species, future experiments will be needed to determine whether these differences are due to the predicted code used to infer optimality in mice or the differences in timing and cell division that might make other mechanisms more prevalent during this transition*** *(Svoboda, Franke et al. 2015). Together, these results indicate that codon optimality mediated control of mRNA stability is a conserved mechanism to regulate differential mRNA stability during the MZT across animals.*

- Additional input/suggestion from other advisor contacted on the relative contribution from codon vs amino acid identity:

'There is some codon effect (Fig 1D) as for some amino acids, there appear to be good and bad codons (though the effects are modest when compared to Presnyak et al. in terms of R value). However, for many amino acids, either all codons are good or all codons are bad - this can be most simply extracted by visual analysis of the same Figure 1D. If all codons are good, then this suggests that the amino acid is relevant, not the codon. The analysis in Figs 4B and 4C is confusing to me but in principle expresses this idea. If you look at where codons cluster in Figures 4B and C it is clear that the majority of codons encoding a given amino acid have similar net effects on CSC (i.e.

Leucine is always mildly destabilizing at best or largely destabilizing at worst). Furthermore, looking at Figure 4D, we find the correlation with amino acid identity to be even greater in range than codon identity. We think it would be better and more simply discussed by colouring the codons by amino acid in Fig 1D. Also, the amino acid effect seems stronger and thus would be better dealt with more carefully and throughout the text. In conclusion there seems to be evidence of both effects; however, the amino acid effects are stronger and more interesting. They're certainly deserving of more discussion than the two paragraphs devoted on page nine of the manuscript. I think if the authors were more willing to be open to these ideas, they would write a clearer manuscript that better summarizes their data, rather than forcing it to look like Presnyak et al.'

Authors: We appreciate the reviewers feedback. We have included the color identity of the codon in figure 1D. While we agree that there tends to be a consistent effect of codons for the same amino acid and this is very interesting. Without further extensive experimental support it would be hard to conclude that, if all the codons for the same amino acid are the same then the effect is the amino acid and not from the codon. We also agree with the reviewer that figure 4 (homeostasis) is also strongly suggesting that the amino acid is playing a role, in agreement with the analysis done during early development. In sum, we believe it is more complex and both tRNA and amino acid are likely to contribute. However it is fair to speculate that if the effect is exclusively dependent on the amino acid, then all the codons encoded for a given amino acid should have the same optimality (Fig 6). However, while all 6 codons for Leucine are Non optimal, we clearly see that UUA and UUG are more destabilizing and; CUG and CUC are the weakest (Fig 1 and 6). In addition, Mishima et. al 2016 has recently showed that silent codon substitutions of CUG to CUA increases destabilization. In our data, CUA also has stronger destabilization effect (Lower CSC and also Lower rCSI) than CUG (Fig 1d and f).
We have added additional text and emphasize the potential importance of amino acid optimality

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"..... influencing mRNA stability when they are decoded. ***This observation adds a novel layer of complexity into the codon optimality, where actually the encoded amino acid might affects the stability of the translated mRNA;*** *future experiments will be needed to uncouple codon and amino acid optimality."*

In page 11

".... folding of highly expressed genes (Akashi, 1994, Akashi & Gojobori, 2002, Drummond & Wilke, 2008, Ikemura, 1982, Kudla et al., 2009, Novoa & Ribas de Pouplana, 2012). ***The amino acid optimality code (Fig. 6) provides an alternative perspective on sequence changes between paralogs in evolution and human disease. Individual gene sequence might be determined not only by the function of the protein but also by its optimality with respect to mRNA stability and translation efficiency potentially driving sequence evolution.*** *Based on the strong effect of codon optimality on mRNA stability ...."*.

"While we observe a correlation between tRNA levels and the extremes of 12 optimality, it is likely that this code integrates multiple inputs of translation from tRNA availability and accuracy (Akashi, 1994, Hussmann et al., 2015, Ishimura et al., 2014), tRNA modification (Gustilo et al., 2008, Novoa et al., 2012), to peptide bond formation, ***amino acid identity****, and other factors influencing the translocation rate and ultimately elongation (Baragana et al., 2015, Faller et al., 2015). Future studies will be needed to determine how the ribosome or accessory proteins decode codon optimality (Richter & Coller, 2015), and how this code is regulated across cellular transitions, tissues and pathological states.*

Bazzini, A. A., M. T. Lee and A. J. Giraldez (2012). "Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish." Science **336**(6078): 233-237.
Park, J. E., H. Yi, Y. Kim, H. Chang and V. N. Kim (2016). "Regulation of Poly(A) Tail and Translation during the Somatic Cell Cycle." Mol Cell **62**(3): 462-471.
Svoboda, P., V. Franke and R. M. Schultz (2015). "Sculpting the Transcriptome During the Oocyte-to-Embryo Transition in Mouse." Curr Top Dev Biol **113**: 305-349.

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Corresponding Author Name: Antonio J Giraldez
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-94699

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?	The number of embryos used in each study was chosen based on previous experience (RNA-seq) or preliminary data (Reporter library, Figure 1). For the Reporter library, we found that 25 injected embryos presented high complexity. Materials and Methods, "Reporter library injection and sample preparation".
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded from the analysis. Only one sample was discarded for low quality reasons after sequencing. Materials and Methods, "Reporter library analysis".
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.	Random set of embryos were used from several pair-wise natural crosses. The pairs were randomly selected among more than 1500 couples.
For animal studies, include a statement about randomization even if no randomization was used.	
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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
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes, all the tests are indicated in the text

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).	NA
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.	Danio Rerio, TLF, AB, embryos Xenopus tropicalis, N (Nigeria), embryos
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	These experiments are compliant with IACUC approved protocols and procedures
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	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 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	For all the unpublished RNA-sequencing data, we are currently depositing in 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).	
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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.	NA