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Inactivation of the type I interferon pathway reveals long double stranded RNA-mediated RNA interference in mammalian cells

Pierre V Maillard, Annemarthe G Van der Veen, Safia Deddouche-Grass, Neil C Rogers, Andres Merits and Caetano Reis e Sousa

Corresponding author: Caetano Reis e Sousa & Pierre Maillard, The Francis Crick Institute

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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   18 July 2016

Thanks for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you, but I have now received comments from the two referees on your manuscript.

As you can see below, both referees find the analysis well done and the data conclusive. A concern raised by referee #1 is that we gain too limited mechanistic insight into how the IFN suppresses the dsRNAi pathway. However, referee #2 also highlights the importance of the findings. While further mechanistic insight into the process would of course be nice I also find that we don't such insight at this stage.

Given the comments from the referees, I would like to ask you to submit a suitably revised manuscript. As mentioned above we don't need any further mechanistic insight, but I would be keen to here you response to referee #1 point 2. The statistical analysis also has to be sorted out. Referee #2 raises a number of constructive points that I am sure that you must have considered and are in a position to respond to. I am available to discuss the specifics further.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the

conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

In this work Maillard et al have examined the role of dsRNAi in antiviral defense in mammalian cells. The work expands on an article from 2013 by Maillard (Science 342:235) demonstating that RNAi is active as an antiviral mechanism in mammalian cells. The idea behind the work is interesting, and the authors try to prove that lack of type I IFN uncovers a role for dsRNAi in antiviral defense. Although the data presented in the present work is well designed, and the conclusions drawn by the authors are generally supported by the data, the work appears underdeveloped.

1. The results are very descriptive, and lack mechanistic explanation for how IFN/ISGs suppress sequence-specific responses to long dsRNAs.

2. Critically, data are missing on the role/impact of endogenous dsRNAi in antiviral defense in Ifnar-/- cells/mice.

3. Throughout, the lack of statistical analysis of the data weakens the conclusions.

Referee #2:

Review of EMBOJ-2016-95086

Maillard et al examine the presence of a functional RNAi pathway in differentiated mammalian cells after inactivating perhaps more dominant innate immune pathways regulated by PRRs or IFN. The authors use long dsRNA as a probe and show dsRNA specificity for repression of GFP expression using a flow cytometry-based assay. The results and controls support the existence of a functional RNAi pathway and cells "immunized" with dsRNA can inhibit replication of an SFV derivative with the target sequence. The paper is well written and the conclusions tempered where appropriate. What is not clear is whether or not this pathway actually plays an important role in mammalian antiviral protection since it does not appear to be sufficiently strong unless cells are first primed with dsRNA. Given the rather polarized views regarding functional RNAi in mammalian cells, a few additional experiments might be in order.

Major points:

1. Can the authors actually show that target-specific small RNAs of the appropriate size are present in the dsRNA treated cells?
2. Although when taken together the control experiments support the authors' thesis, it would be reassuring and bolster their argument for sequence specificity to show that a different, non-targeted fluorescent reporter (say RFP or a recoded GFP to ablate the target homology) with otherwise the same mRNA structure does not exhibit decreased expression by the dsRNA that inhibits GFP expression. Off target effects of transfected nucleic acids are always a concern and a non-targeting dsRNA of markedly different sequence may not be a sufficiently rigorous control.

Minor points:

1. For most of the experiments, silencing induced by dsRNA was not restricted to a subset and the entire transfected population shifted towards the left, with one peak. However, some of the data

(Fig.3B, Fig.4D), there are two distinct peaks for dsRNA-GFP transfection (one shifted to low expression, the other overlapped with Mock). Does this imply that the efficacy of silencing can be affected by other factors?

2. For the Fig. 5A western blot, it might be desirable to add a lane with parental Mavs-/- cells probed with Ago2 antibody, to show the levels of restored expression of Ago2. Theoretically, the level of Ago2 should be critical for the shift.

3. There are several possibilities for the failure to observe RNAi after viral infection of IFN-deficient cells: (1) VSRs obscure RNAi effects; (2) dsRNAi is not robust enough to provide effective antiviral effects. Probably beyond the scope of this initial report, but finding a natural context where the IFN response is suppressed and this RNAi type of response takes over would be ideal. But this might be very difficult to find if selection pressure for antiviral protection has shifted to the IFN and protein based innate immune responses (especially those focused on dsRNA sensing). Thus while the RNAi factors have been co-opted in vertebrates for other purposes, it might not be too surprising that there is some low level "vestigial" activity. A prediction/question that could be tested: If you test VSR deficient viruses, would you expect to see less efficient replication in parental Mavs-/- cells, compared to Ago2-/- Mavs-/- cells?

4. There are many "representative" data panels, rather than showing the average, error bars (standard deviation) and statistical measures of significance. Figure 1G, for example, lacks error bars. Given that the effects are rather subtle in some of the experiments, it is important to give readers (and reviewers) an idea of how much variability is observed between replicate experiments.

1st Revision - authors' response   02 September 2016

Referee #1

In this work Maillard et al have examined the role of dsRNAi in antiviral defense in mammalian cells. The work expands on an article from 2013 by Maillard (Science 342:235) demonstating that RNAi is active as an antiviral mechanism in mammalian cells. The idea behind the work is interesting, and the authors try to prove that lack of type I IFN uncovers a role for dsRNAi in antiviral defense. Although the data presented in the present work is well designed, and the conclusions drawn by the authors are generally supported by the data, the work appears underdeveloped.

1. The results are very descriptive, and lack mechanistic explanation for how IFN/ISGs suppress sequence-specific responses to long dsRNAs.

We agree with the reviewer that our study does not explain how ISGs suppress long dsRNAi in mammalian cells. However, the mere existence of the process has been so controversial that we felt that, in a first report, it would be most important to thoroughly document it and investigate its possible functional consequences. We hope to investigate the mechanistic underpinnings of our observations in subsequent studies.

2. Critically, data are missing on the role/impact of endogenous dsRNAi in antiviral defense in Ifnar-/- cells/mice.

We thank the reviewer for suggesting that we expand on this aspect, which was missing from our original submission. We have performed a series of experiments that we have now included as part of Appendix Figure S7. We have performed infections with reovirus, Sindbis virus, influenza A virus and encephalomyocarditis and have failed to find evidence for an impact of dsRNAi on virus accumulation. The possible implications of these data are mentioned in the discussion of our manuscript.

3. Throughout, the lack of statistical analysis of the data weakens the conclusions.

We have previously presented flow cytometry data from single representative experiments. This is customary in FACS analysis because such data are not easily amenable to pooling between experiments. For example, median fluorescence intensity values are not absolute and depend on the machine used, its settings and, even if a single machine and identical settings are used, the extent of laser drift between experiments. Nevertheless, we have now been able to pool data from

independent experiments by normalising in each experiment the median fluorescence intensity values from each sample to those of the mock control. We now show bar graphs depicting these pooled data with standard deviations and statistical analysis carried out using two-way ANOVA or unpaired *t*-test (if only 2 conditions are compared). For Figure 6, exact virus titres obtained after infection can vary from one experiment to the next and pooling generates large errors, which are absent when replicate samples within a single experiment are compared. Therefore, we show data for a representative experiment for clarity, yet we added to Appendix Figure S6 a summary of all the data from all independent experiments. These all show the same effect and confirm the robustness of our observations.

Referee #2:

Maillard et al examine the presence of a functional RNAi pathway in differentiated mammalian cells after inactivating perhaps more dominant innate immune pathways regulated by PRRs or IFN. The authors use long dsRNA as a probe and show dsRNA specificity for repression of GFP expression using a flow cytometry-based assay. The results and controls support the existence of a functional RNAi pathway and cells "immunized" with dsRNA can inhibit replication of an SFV derivative with the target sequence. The paper is well written and the conclusions tempered where appropriate. What is not clear is whether or not this pathway actually plays an important role in mammalian antiviral protection since it does not appear to be sufficiently strong unless cells are first primed with dsRNA. Given the rather polarized views regarding functional RNAi in mammalian cells, a few additional experiments might be in order.

Major points:
1. Can the authors actually show that target-specific small RNAs of the appropriate size are present in the dsRNA treated cells?

We now include as part of Fig 4A a Northern blot of total RNA from *Ifnar1*$^{-/-}$ MEFs transfected with dsRNA-*RL* or dsRNA-*GFP*. Using probes specific for either dsRNA-*GFP* or dsRNA-*RL,* we could detect 22-nt cleavage products that co-migrated with Dicer-dependent siRNAs generated in *vitro*. We thank the reviewer for encouraging us to perform this experiment, the results of which have strengthened our manuscript.

2. Although when taken together the control experiments support the authors' thesis, it would be reassuring and bolster their argument for sequence specificity to show that a different, non-targeted fluorescent reporter (say RFP or a recoded GFP to ablate the target homology) with otherwise the same mRNA structure does not exhibit decreased expression by the dsRNA that inhibits GFP expression. Off target effects of transfected nucleic acids are always a concern and a non-targeting dsRNA of markedly different sequence may not be a sufficiently rigorous control.

The referee raises the concern that the effect that we see on GFP expression using dsRNA-*GFP* might be caused by off-target effects restricted to dsRNA-*GFP* but not with dsRNA-*RL*. Yet, we could observe that the dsRNA-*RL* is also processed into siRNAs (see section above and Fig 4A) and, more importantly, the same dsRNA-*RL* is used against SFV-RLuc in the experiments in Fig 6, in which dsRNA targeting GFP is now used as a control. We observe a sequence-specific antiviral activity against SFV-Rluc provided by the cognate dsRNA-*RL* compared to dsRNA-*GFP*. This criss-cross effect is, in our view, the best measure of specificity as it demonstrates clearly that each dsRNA used in the paper has RNAi activity that specifically impacts only the expression of its cognate target.

Minor points:

1. For most of the experiments, silencing induced by dsRNA was not restricted to a subset and the entire transfected population shifted towards the left, with one peak. However, some of the data (Fig.3B, Fig.4D), there are two distinct peaks for dsRNA-GFP transfection (one shifted to low expression, the other overlapped with Mock). Does this imply that the efficacy of silencing can be affected by other factors?

The efficiency of silencing correlates positively with the efficiency of transfection with Cy5 labeled dsRNA (Cy5-dsRNA). For most experiments, the level of transfection was such that the majority of the cells became Cy5^{high} when analysed by flow cytometry. Yet, in some experiments, transfection was less efficient resulting in cells that were Cy5^{low} to Cy5^{high}. Given the reviewer's comment we reanalyzed the data from Fig. 3B using a more stringent gate to select more specifically the Cy5^{high} cells that had been transfected most efficiently. We replaced the plots of Fig. 3B with the analysis obtained with this more stringent gating strategy. The reviewer also mentioned the plots of Fig 4 D (now part of Fig 4 C). For those analyses, a more stringent gating was not appropriate as this resulted in a very low number of cells within the gate. We therefore left the panels of Fig.4 C unchanged but added a note in the figure legend to provide this information to the reader.

2. *For the Fig. 5A western blot, it might be desirable to add a lane with parental Mavs-/- cells probed with Ago2 antibody, to show the levels of restored expression of Ago2. Theoretically, the level of Ago2 should be critical for the shift.*

We have repeated the Western blot including, as requested, the parental *Mavs$^{-/-}$* cells and incorporated it into Fig 5A. We probed the membrane first with Ago2 antibody and then stripped it off and probed it with an anti-HA antibody. We found that the level of endogenous Ago2 in parental *Mavs$^{-/-}$* cells is similar to the level of Ago2 detected in the 2 *Mavs$^{-/-}$ Ago2$^{-/-}$* clones complemented with HA-mAgo2 WT. The sequence-specific gene silencing is observed in these 3 cell lines but not in cells that do not express Ago2 (*Mavs$^{-/-}$ Ago2$^{-/-}$* transduced with empty vector). The sequence-specific effect is also abolished in cells that express a catalytic mutant of Ago2 (HA-mAgo2 D597A) either at similar (lane 4) or much higher levels (lane 7) than endogenous Ago2 parental *Mavs$^{-/-}$* cells.

3. *There are several possibilities for the failure to observe RNAi after viral infection of IFN-deficient cells: (1) VSRs obscure RNAi effects; (2) dsRNAi is not robust enough to provide effective antiviral effects. Probably beyond the scope of this initial report, but finding a natural context where the IFN response is suppressed and this RNAi type of response takes over would be ideal. But this might be very difficult to find if selection pressure for antiviral protection has shifted to the IFN and protein based innate immune responses (especially those focused on dsRNA sensing). Thus while the RNAi factors have been co-opted in vertebrates for other purposes, it might not be too surprising that there is some low level "vestigial" activity. A prediction/question that could be tested: If you test VSR deficient viruses, would you expect to see less efficient replication in parental Mavs-/- cells, compared to Ago2-/- Mavs-/- cells?*

As suggested by the reviewer, an impact of RNAi on virus infection might be more easily revealed using VSR-deficient viruses. We therefore tested influenza virus ΔNS1 because NS1 was shown to have VSR activity in Drosophila cells[1] and inhibit production of siRNA from dsRNA substrate in plants[2-4] and human cells[5]. However, we did not detect a difference in the replication of influenza ΔNS1 (or parental virus) in cells displaying or not a functional RNAi pathway. Influenza virus is negatively stranded and may not produce sufficient amounts of dsRNA[6,7]. Therefore, we also tested a positively-stranded picornavirus, EMCV, lacking the L protein (which might constitute another possible VSR on the basis that it inhibits IFN responses). Again, we did not see an effect of antiviral RNAi with EMCV ΔL. We have included these data, together with data from other virus infections experiments, as part of Appendix Figure 7 and discuss their implications in the text.

4. *There are many "representative" data panels, rather than showing the average, error bars (standard deviation) and statistical measures of significance. Figure 1G, for example, lacks error bars. Given that the effects are rather subtle in some of the experiments, it is important to give readers (and reviewers) an idea of how much variability is observed between replicate experiments.*

Please see our answer to comment 3 of reviewer 1.

References.

1.   Li, W. X. *et al.* Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proc Natl Acad Sci U S A* **101,** 1350–1355 (2004).
2.   Delgadillo, M. O., Sáenz, P., Salvador, B., García, J. A. & Simón-Mateo, C. Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. *J Gen Virol* **85,** 993–999 (2004).

3.   Bucher, E., Hemmes, H., de Haan, P., Goldbach, R. & Prins, M. The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* **85,** 983–991 (2004).

4.   de Vries, W., Haasnoot, J., Fouchier, R., de Haan, P. & Berkhout, B. Differential RNA silencing suppression activity of NS1 proteins from different influenza A virus strains. *J Gen Virol* **90,** 1916–1922 (2009).

5.   Kennedy, E. M. *et al.* Production of functional small interfering RNAs by an amino-terminal deletion mutant of human Dicer. *Proceedings of the National Academy of Sciences* (2015). doi:10.1073/pnas.1513421112

6.   Weber, F., Wagner, V., Rasmussen, S. B., Hartmann, R. & Paludan, S. R. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J. Virol.* **80,** 5059–5064 (2006).

7.   Pichlmair, A. *et al.* Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J. Virol.* **83,** 10761–10769 (2009).

Accepted	12 September 2016

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #2 and the comments are provided below. As you can see the referee appreciates the introduced changes and balanced discussion. I am therefore very pleased to accept the manuscript for publication.

REFEREE REPORT

Referee #2:

The authors have adequately addressed our main concerns. Kudos for doing the VSR deficient virus experiment. Not seeing any difference on the surface sinks the argument that this is an ancient "backup" system. However, I'd be hard pressed to fully believe this negative result given the super artificial (but state of the art) cell culture context used. Their language on why they could be missing antiviral dsRNAi reads appropriately.

Bottom line: This is a result awaiting functional impact, but well reasoned and controlled. It opens up future work to look for a context where this pathway makes a difference.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Caetano Reis e Sousa	
Journal Submitted to: The EMBO Journal	
Manuscript Number: EMBOJ-2016-95086	

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).

B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For flow cytometry experiments, the data represents a sample size of 10 000 cells according to standard procedure. Information found in the Materials and Methods, section "dsRNA or siRNA transfection and flow cytometry", p.27
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?	NA
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.	NA
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.	NA
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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?	NA
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).	The description of the antibodies used in this study is mentionned in the Materials and Methods, section "protein analysis", pp.28-29
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The information on cell lines are mentionned in the Materials and Methods, section "cells", p.21

* 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

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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
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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).	NA
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. 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	NA
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.	NA

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.	No