

Manuscript EMBO-2016-96025

A G1-like state allows HIV-1 to bypass SAMHD1 restriction in macrophages

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Review timeline:

Submission date:	08 November 2016
Editorial Decision:	05 December 2016
Revision received:	12 December 2016
Accepted:	21 December 2016

Editor: Karin Dumsrei

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

05 December 2016

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by three good experts in the field and their comments are provided below.

As you can see from the comments, the referees find the analysis interesting and suitable for publication here. They raise a few relative minor concerns that shouldn't involve too much additional work to sort out.

Let me know if we need to discuss anything further.

REFeree REPORTS

Referee #1:

The authors noted that HIV-1 transduction efficiency was greater in macrophages cultured in fetal calf serum than in human serum. Differential expression profiling (FCS vs HS) revealed differences in cell cycle regulators that were confirmed by western blots for cyclins A, D, E, CDK1, MCM2, p27, geminin, and E2F6, though cells remained blocked before entry into S phase. The cell cycle effects were associated with SAMHD1 phosphorylation, and, via a number of convincing experiments, the cell cycle-dependent, Raf/MEK/ERK-dependent, SAMHD1 phosphorylation was shown to explain the effects of the FCS on HIV infectivity. Individual macrophages were assessed by microscopy and it was shown that transduction correlated with cell cycle markers for G1;

correlation was lost with Vpx or other controls. Experimental results were replicated using macrophages from WT and SAMHD1 KO mice. The mouse experiments also confirmed that G1 macrophages are not a tissue culture artifact. HDAC inhibitors blocked the effect of FCS.

This is a very important manuscript that clearly explains how HIV-1 is capable of infecting macrophages, despite the fact that the virus lacks a protein, such as Vpx, that counteracts the antiviral effect of SAMHD1. We have only trivial suggestions to improve the manuscript.

1. Page 3, line 2: The statement that myeloid and resting T cells express SAMHD1 is kind of irrelevant, since it implies that other cell types do not express it. The second sentence about SAMHD1 phosphorylation in dividing cells then is kind of confusing.
2. Page 4, line 14: The relevance to the experiments here of the HIV-1 capsid mutants is not clear. It seems an irrelevant distraction.
3. Page 4, line 16: With respect to the charcoal stripping, did the authors ever try to mix the two sera together to see which phenotype is dominant? Is the sera heat-inactivated? Does heat kill the activity?
4. Page 4, line 22: Why call them stimulated and unstimulated? It would be clearer to the reader if they were simply called FCS vs HS.

Referee #2:

Mlcochova and colleagues examined why HIV-1 is capable of infecting macrophages although these express SAMHD1 and the virus does not express an antagonist of this restriction factor. They show that parts of the macrophages are in a G1-like phase that is associated with expression of various cell cycle associated proteins including CDK1 that phosphorylates and hence inactivates SAMHD1. The authors also provide evidence that these events are induced by FCS treatment via stimulation of the Raf/MEK/ERK cascade. Finally, the authors provide evidence that HDAC inhibitors prevent HIV-1 infection of macrophages in a SAMHD1 dependent manner.

The experiments are for most part well performed and controlled. One limitation of the study is that the novelty of the findings seems somewhat limited. It is known that macrophages become susceptible to HIV-1 infection after inactivation of the restriction factor SAMHD1 by CDK1. Furthermore, Badia and colleagues have recently shown that cell cycle proteins affect SAMHD1-mediated HIV-1 restriction in macrophages. This paper is mentioned in the discussion but not properly cited. Thus, the major novel aspects here seem to be the observation that FCS activates CDK1 via the Raf/MEK/ERK pathway and the inhibitory effect of HDAC inhibitors. Thus, should be clarified. Furthermore, some flaws in the manuscript need to be fixed.

Specific points

1. Abstract: the authors state "SAMHD1 activation by phosphorylation". However, as correctly stated elsewhere in the manuscript phosphorylation of SAMHD1 impairs its anti-HIV activity. To avoid confusion statement should be checked for accuracy throughout (see e.g. pg. 11, line 17 "inactive unphosphorylated SAMHD1").
2. The authors generally determined percentages of infected cells. For most part that's fine but the should examine whether induction of the G1-like phase is also associated with higher levels of infectious virus production by the infected cultures.
3. The infection rates in unstimulated macrophages are much higher in the experiment shown in Figure 2G than in 1A. This discrepancy should be discussed.
4. Did none of the inhibitors used to generate the data shown in Figure 2 show cytotoxic effects?
5. The inducing factor remains elusive and it seems surprising that it is found in FCS but not in human sera. The authors mention that they "could not reproduce the effect of FCS using human cord

blood derived serum". Further detail should be provided and they should discuss possible reasons for the differential effects of human and bovine sera (preparation, species-specific differences,...?). Obviously, identification of the factor would clearly increase the significance of the study.

6. At several places commas and spaces are missing.

Referee #3:

This is a beautiful and focused study addressing a long standing question in the field. They identified a population of non-dividing monocytes derived macrophages and resident macrophages that expresses markers of G1 as opposed to G0 macrophages. Transition from G0 to G1 overcomes the SAMHD1 restriction activity by inducing its phosphorylation at T592. The experiments are smartly designed, consistent, and fully support the conclusions. The study will certainly represents a forward step towards our understanding of macrophages as target for HIV-1 and their role in HIV-1 pathogenesis.

This reviewer is highly supportive for publication in The EMBO Journal.

Straightforward study. The experiments are well designed and appropriate. They fully support the conclusions.

I therefore have no additional experiments to ask for.

1st Revision - authors' response

12 December 2016

Referee #1:

The authors noted that HIV-1 transduction efficiency was greater in macrophages cultured in fetal calf serum than in human serum. Differential expression profiling (FCS vs HS) revealed differences in cell cycle regulators that were confirmed by western blots for cyclins A, D, E, CDK1, MCM2, p27, geminin, and E2F6, though cells remained blocked before entry into S phase. The cell cycle effects were associated with SAMHD1 phosphorylation, and, via a number of convincing experiments, the cell cycle-dependent, Raf/MEK/ERK-dependent, SAMHD1 phosphorylation was shown to explain the effects of the FCS on HIV infectivity. Individual macrophages were assessed by microscopy and it was shown that transduction correlated with cell cycle markers for G1; correlation was lost with Vpx or other controls. Experimental results were replicated using macrophages from WT and SAMHD1 KO mice. The mouse experiments also confirmed that G1 macrophages are not a tissue culture artifact. HDAC inhibitors blocked the effect of FCS.

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1. Page 3, line 2: The statement that myeloid and resting T cells express SAMHD1 is kind of irrelevant, since it implies that other cell types do not express it. The second sentence about SAMHD1 phosphorylation in dividing cells then is kind of confusing.

Response: We agree with this point and thank the reviewer for pointing it out. We have now removed the sentences in question on page 3.

2. Page 4, line 14: The relevance to the experiments here of the HIV-1 capsid mutants is not clear. It seems an irrelevant distraction.

Response: we know that cofactor interactions impact RT and are important for efficient HIV infection in macrophages. We therefore wished to test the hypothesis that the effect of FCS might be due to regulation of host co-factors sensitive to mutations in capsid. Even though this did not prove to be the case we believe that these are important negative data and would like to keep them in manuscript as long as the editor agrees.

3. Page 4, line 16: With respect to the charcoal stripping, did the authors ever try to mix the two sera together to see which phenotype is dominant? Is the sera heat-inactivated? Does heat kill the activity?

Response: We did mix the sera and found that the FCS phenotype is dominant, see figure below which we have now included as Extended view Figure 1A and added relevant text (page 4 lines 86-89).

The sera used were all heat inactivated and boiling FCS failed to abrogate the phenotype. We have added a figure to extended view as well as text in the results section to reflect this addition (page 4 lines 86-89).

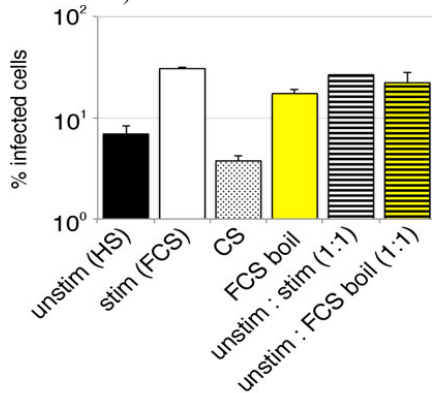


Figure: Monocyte derived macrophages (MDM) were differentiated and cultured in RPMI complemented with MCSF and 10% Human Serum (UNSTIM) and changed at day 3 for 10% Fetal Calf Serum (STIM), 10% charcoal stripped FCS (CS), 10% FCS boiled for 5min (FCS boil), or 10% of 1:1 mixed HS:FCS (unstim:stim), HS:FCS boil (unstim:FCS boil) and infected with VSV-G pseudotyped HIV-1 expressing GFP. Percentage of infected cells was quantified 48h post-infection by FACS.

4. Page 4, line 22: Why call them stimulated and unstimulated? It would be clearer to the reader if they were simply called FCS vs HS.

Response: We debated this point for some time but arrived at ‘stimulated versus unstimulated’ because FCS stimulates the canonical Raf/MEK/ERK pathway to induce cell cycle entry, thereby making the figures easier to understand for the reader. As the other 2 reviewers did not comment we would prefer to keep as is unless the editor decides otherwise.

Referee #2:

Mlcochova and colleagues examined why HIV-1 is capable of infecting macrophages although these express SAMHD1 and the virus does not express an antagonist of this restriction factor. They show that parts of the macrophages are in a G1-like phase that is associated with expression of various cell cycle associated proteins including CDK1 that phosphorylates and hence inactivates SAMHD1. The authors also provide evidence that these events are induced by FCS treatment via stimulation of the Raf/MEK/ERK cascade. Finally, the authors provide evidence that HDAC inhibitors prevent HIV-1 infection of macrophages in a SAMHD1 dependent manner.

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Response: We are pleased that the reviewer feels 2 major aspects of the paper are novel. However, we disagree about novelty related to permissivity of macrophages and cell cycle associated proteins. We show that early cell cycle entry (from G0 to G1) is associated with dramatic changes in SAMHD1 dependent permissivity to HIV at the single cell level. This central finding in our paper was realised by rigorous quantification of co-staining in tens of thousands of cells, with a number of controls. The focus of the Badia et. al. paper was on the inhibitory effect of GMCSF on permissivity of macrophages acting via CDK1 and cyclin D2. We apologise for not citing the paper correctly in the bibliography, which we have now rectified (line 324). Thus our data are highly novel, explaining

how HIV replicates without the need for a SAMHD1 antagonist in both monocyte derived and tissue resident macrophages.

Furthermore, some flaws in the manuscript need to be fixed. Specific points

1. Abstract: the authors state "SAMHD1 activation by phosphorylation". However, as correctly stated elsewhere in the manuscript phosphorylation of SAMHD1 impairs its anti-HIV activity. To avoid confusion statement should be checked for accuracy throughout (see e.g. pg. 11, line 17 "inactive unphosphorylated SAMHD1").

Response: We thank the reviewer for pointing this mistake out – we have now corrected it and checked the paper.

2. The authors generally determined percentages of infected cells. For most part that's fine but the should examine whether induction of the G1-like phase is also associated with higher levels of infectious virus production by the infected cultures.

Response: we have measured infectious virus production in infected cultures and we have now presented these data as Extended view Figure 1D.

3. The infection rates in unstimulated macrophages are much higher in the experiment shown in Figure 2G than in 1A. This discrepancy should be discussed.

Response: the discrepancy is due to donor variation in absolute infection rate and the fact that we have used a representative donor for Fig 2G. The donor variability among 12 donors is demonstrated in Extended view figure 1B. In this figure one can find multiple donors with similar susceptibility as in Fig 2G. We have added text explaining that there is donor variability (lines 79-80).

4. Did none of the inhibitors used to generate the data shown in Figure 2 show cytotoxic effects?

Response: We thank the reviewer for this question. All inhibitors were carefully titrated on MDM and non-toxic, effective concentrations of compounds were determined and used for further experiments. The lack of cytotoxic effect is evidenced by the fact that SAMHD1 degradation rescued viral titre completely, as documented in the Figure below.

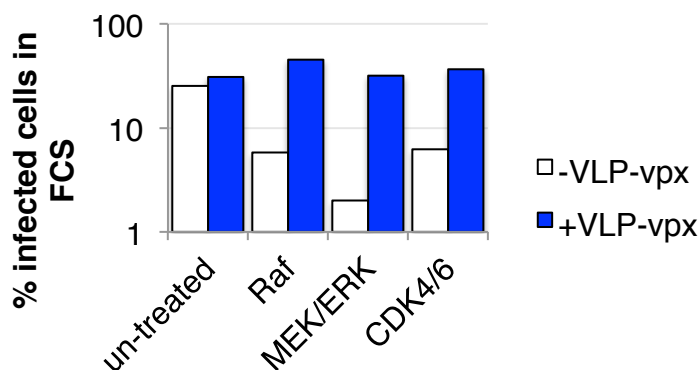


Figure: Stimulated MDM were treated with inhibitors of RAF (2mM); MEK1/2 (AS-703026, 1mM); and CDK4/6 (1mM) 18h before infection. MDM were coinfecting with VSV-G HIV-1 GFP and SIVmac Virus Like Particles containing vpx (VLP-vpx, degrades SAMHD1). Percentage of infected cells were quantified by FACS 48h post-infection.

5. The inducing factor remains elusive and it seems surprising that it is found in FCS but not in human sera. The authors mention that they "could not reproduce the effect of FCS using human cord blood derived serum". Further detail should be provided and they should discuss possible reasons for the differential effects of human and bovine sera (preparation, species-specific differences,...?). Obviously, identification of the factor would clearly increase the significance of the study.

Response: We agree that identification of the factor is important and we are undertaking an extensive study, but we would like to emphasise that the FCS and HS were primarily used as a tool to study macrophages in G1 and G0. Data from 19 human cord blood derived serum samples are shown below. No statistical significance was detected. We have also now discussed possible differences between human and foetal calf serum in the text (lines 314-320).

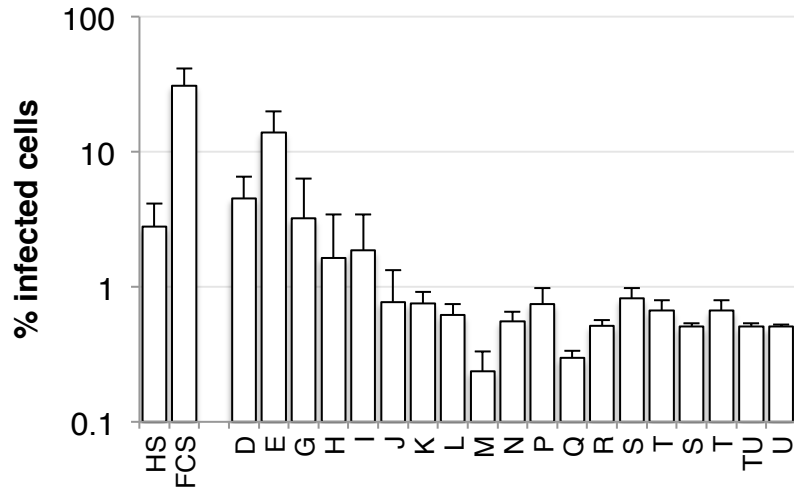


Figure: MDM were cultured in human serum, FCS or human cord blood derived serum (individual donors in capital letters) for 3 days and infected with VSV-pseudotyped HIV-1GFP. Percentage of infected cells was determined 48h postinfection by FACS.

6. At several places commas and spaces are missing.

Response: we have now proof read the paper again paying particular attention to this.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ravindra Gupta

Journal Submitted to: EMBO J

Manuscript Number: EMBOJ-2016-96025

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 χ^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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We used cells from mouse peritoneum and were able to isolate thousands per animal. We chose 3 control and 3 knockout animals for each experiment
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.	NO
For animal studies, include a statement about randomization even if no randomization was used.	We did not randomise
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
4.b. For animal studies, include a statement about blinding even if no blinding was done	The operator was blinded to whether mice were WT or KO for SAMHD1
5. For every figure, are statistical tests justified as appropriate?	YES - see page 27 onwards
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 - see figs and error bars
Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents**USEFUL LINKS FOR COMPLETING THIS FORM**
<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>
<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>
<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun>
<http://datadryad.org>
<http://figshare.com>
<http://www.ncbi.nlm.nih.gov/gap>
<http://www.ebi.ac.uk/ega>
<http://biomodels.net/>
<http://biomodels.net/miriam/>
<http://ij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	yes this has been done and is in the methods page 17-22
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 - We used primary cells for all experiments

* 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.	Methods: page 17-22
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	page 22
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 we have complied

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	National Research Ethics Service through The Joint UCL/UCLH Committees on the Ethics of Human Research (Committee Alpha) 2nd of December 2009. Reference number 06/Q0502/92.
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.	page 23
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.	None
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	Microarray data are available in the ArrayExpress database under accession number E-MTAB-2985 for stimulated MDM (differentiated in FCS) and E-TABM-1206 for all other cell types presented in this study
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).	Microarray data are available in the ArrayExpress database under accession number E-MTAB-2985 for stimulated MDM (differentiated in FCS) and E-TABM-1206 for all other cell types presented in this study
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 <i>Shewanella oneidensis</i> 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 Biomedel (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
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