EMBO reports

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Alternative lengthening of human telomeres is a conservative DNA replication process with features of break-induced replication

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Sarantis Gagos, Biomedical Research Foundation of the Academy of Athens

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Submission date:	04 August 2016
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Editor: Esther Schnapp

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 24 August 2016

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports on it that is pasted below.

As you will see, all referees acknowledge that the findings are interesting and support publication of the study here. They only have a few comments that I think can and should all be addressed. Please let me know if you estimate the revisions to take more than a few weeks. I suggest that you submit the revised manuscript by mid September in order to publish the paper as soon as possible.

Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second, short round of review.

Given the 3 main figures, we will publish the study as a short report. For short reports, the revised manuscript should not exceed 25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. The entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the

respective figure legends. This information must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

For our website we also need A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The manuscript by Roumelioti et al. reports that break-induced replication (BIR) mediates alternative lengthening of telomeres (ALT) in human cells lacking telomerase activity. Whilst BIR has been previously implicated in maintaining ALT telomeres in yeast, the present study provides for the first time evidence that a similar mechanism acts in human cells. These results are novel, provide mechanistic insight into ALT telomere hoemostasis and are therefore suitable for publication in EMBO Reports.

Remarkably, a significant percentage (approx. 11%) of telomeres in U2OS cells, an ALT cell line, replicate conservatively through the BIR pathway. The authors report that human ALT largely relies on POLD3 and POLD4 subunits of polymerase delta, which were previously established by Halazonetis laboratory as key players in collapsed replication fork repair (Costantino et al., Science

343, 2015). Moreover, replication stress induced by cyclin E overexpression increased the frequency of conservatively-replicated telomeres, probably due to higher rates of fork collapse within telomeres and their BIR-mediated restart. Overall, this is a timely and well-executed study, particularly relevant to oncogene-induced replication stress in ALT cells and tumours.

The triple-FISH protocol, described here for the fist time, appears effective in differentiating semi-conservative from conservative telomere replication. The authors recognise its technical limitations (i.e. only a fraction of telomeres can be reliably analysed). Nevertheless, to my knowledge this pioneering approach is the only means for detection of conservative, BIR-mediated telomere replication in human cells. In future studies, it will be interesting to determine the frequency of BIR- versus telomerase-dependent telomere elongation events in telomerase-proficient cell lines.

Minor points:

1. The "all conservative" category in Fig. 2B and corresponding figure legend is confusing and should be better defined (and also linked to the images in Fig. 2A)
2. Exclusion of T-SCE (page 9, top) should be also clarified, preferably with a diagram or representative images similarly to Fig. 2A.
3. An alternative term to "pathognomonic" should be used in the text.

Referee #2:

The paper "Alternative lengthening of human telomeres is a conservative DNA replication process with features of break-induced replication" by Fani-Marlen Roumelioti et al presents a very significant breakthrough in our understanding of the molecular mechanisms of Alternative Telomere Lengthening (ALT), a pathway responsible for telomere maintenance in approximately 15% of cancers. In particular, the authors used state of the art methods, including telomeric in situ hybridization involving three consecutive staining steps. Using these methods, the authors found the presence of conservatively replicated telomeric DNA in telomerase-negative cancer cells. Another important finding of this study was that depletion of PolD3 and PolD4, two subunits of human polymerase delta that are known to be essential for BIR, reduced the frequency of conservatively replicated telomeric DNA ends and led to shorter telomeres and to the increase of chromosome end-to-end fusions. Together, these two findings confirm two important hypotheses that were proposed based on multiple yeast studies, but were never tested directly in human cells: (i) that BIR is responsible for ALT in humans; and (ii) that BIR in human cells proceeds via conservative DNA synthesis. Overall, the new insights into the mechanisms of ALT resulting from this paper represents a very important development in this field. Because the interest to this topic is very high, I expect that this paper will be frequently cited and will also stimulated further research in various areas including human oncology, DNA repair, replication, and recombination.

Specific comments.

1. Fig. 3A and the text on page 7.
Based on the data presented in this figure, the authors proposed that depletion of POLD3 and POLD4 decrease the percent of conservative synthesis at telomeres. Is this reduction statistically significant? What kind of statistics the authors used to confirm this idea? Is the percent of conservative synthesis shown in this figure represent an average from several experiments or the percent based on the results of all experiments combined together?

2. Figure 3B and the text on page 7
Are the results suggesting that Q-FISH differs between Ctrl and POL3D and between Ctrl and POLD4 is supported statistically? This my question is based on seemingly overlapping SDs between Ctrl and POLD3 and between Ctrl and POLD4. Which statistical methods were used to distinguish between the control and experimental conditions?

3. Figure 3C and the text on page 7.
The frequency of chromosome end-to-end fusions seem to differ significantly between Ctrl and POLD4? Specifically, the SDs obtained for these two experiments overlap. Also, it remains unclear what kind of statistical methods were used to distinguish between these two groups.

Referee #3:

Roumelioti et al. study the mechanism of recombination mediated telomere lengthening (ALT) in mammalian cells. They address two essential in this field questions. First, is the key break induced replication (BIR) repair pathway protein PolD3 (yeast Pol32) needed for ALT, and second, is the fate of newly synthesized strands similar to a regular replication (semiconservative) or BIR (conservative), as studied in yeast. The authors found out that Pol32 is required for ALT and that synthesis mode reminds the one observed previously in BIR in yeast. Moreover they demonstrate that overexpression of cyclin E stimulates BIR indicating that fork breakage could play a role in ALT. Together, the authors provide many insights into the mechanism of ALT.

Concerns:
A control cell line - non ALT is essential to demonstrate that all the events described here are observed only in ALT positive cells.

The authors performed a triple FISH staining to distinguish between conservative and semiconservative mode of newly synthesized DNA inheritance. The results implicate that up to few percent of cells have telomeres built exclusively from new or parental strands, it is hard to understand how would it be possible. For any recombination between telomeres (sister or nonsister) to occur there must be some telomeric sequence made during regular replication that engages in recombination. Thus at least some "semiconservative" part of telomeric sequence must be present on each chromosome end. A comment on this is required.

Are the differences presented in Fig. 3A statistically significant? One of the most sensitive ways to test telomere recombination in ALT cells is to examine the presence of byproducts of recombination, C-circles. It would be beneficial to test the presence of C-circle upon PolD3/4 depletion.

Minor

Fig 2b, please change "all cnsrv" to "total consrv" as all cnrsv indicate category where whole telo is build from new strands.

It would help to explain earlier in the manuscript why the authors use o/e of cyclin E.

1st Revision - authors' response 18 September 2016

Response to Comments of the Referees

Referee #1:

"The manuscript by Roumelioti et al. reports that break-induced replication (BIR) mediates alternative lengthening of telomeres (ALT) in human cells lacking telomerase activity. Whilst BIR has been previously implicated in maintaining ALT telomeres in yeast, the present study provides for the first time evidence that a similar mechanism acts in human cells. These results are novel, provide mechanistic insight into ALT telomere hoemostasis and are therefore suitable for publication in EMBO Reports.

Remarkably, a significant percentage (approx. 11%) of telomeres in U2OS cells, an ALT cell line, replicate conservatively through the BIR pathway. The authors report that human ALT largely relies on POLD3 and POLD4 subunits of polymerase delta, which were previously established by the Halazonetis laboratory as key players in collapsed replication fork repair (Costantino et al., Science 343, 2015). Moreover, replication stress induced by cyclin E overexpression increased the frequency of conservatively-replicated telomeres, probably due to higher rates of fork collapse within telomeres and their BIR-mediated restart. Overall, this is a timely and well-executed study, particularly relevant to oncogene-induced replication stress in ALT cells and tumours.

The triple-FISH protocol, described here for the fist time, appears effective in differentiating semi-conservative from conservative telomere replication. The authors recognise its technical limitations (i.e. only a fraction of telomeres can be reliably analysed). Nevertheless, to my knowledge this pioneering approach is the only means for detection of conservative, BIR-mediated telomere replication in human cells. In future studies, it will be interesting to determine the frequency of BIR- versus telomerase-dependent telomere elongation events in telomerase-proficient cell lines."

We thank the Referee for the overall positive comments. Indeed, the limitations of the triple-FISH protocol were discussed in the manuscript. However, as the Referee states, this novel method is the only method available to monitor conservative DNA replication in human cells. Furthermore, the method reliably identifies conservative replication. Its only limitation is that not all telomeres can be analyzed, because some telomeres are not well-stained through all the three staining steps of the method. However, it is easy to identify the well-stained telomeres. We also thank the Referee for acknowledging that examining the telomeres of telomerase-positive cells should be the subject of future studies. Indeed, our conclusions relate to cells that lack telomerase activity and, therefore, are not dependent on analysis of telomerase-positive cells.

"Minor points:

1. The "all conservative" category in Fig. 2B and corresponding figure legend is confusing and should be better defined (and also linked to the images in Fig. 2A)."

We renamed this category and modified Figs 1, 2 and 3, accordingly. Hopefully, the new terms will be less confusing.

"2. Exclusion of T-SCE (page 9, top) should be also clarified, preferably with a diagram or representative images similarly to Fig. 2A."

T-SCEs are well-defined in the telomere field. They are easily detected in the first step of our staining protocol, which is a conventional denaturing FISH staining. Thus, we are not sure that describing T-SCEs (which were excluded in our study) adds needed clarity to our manuscript. We hope that the Referee will agree.

"3. An alternative term to "pathognomonic" should be used in the text."

We rewrote the sentence containing this term and now do not use the term "pathognomonic".

Referee #2:

"The paper "Alternative lengthening of human telomeres is a conservative DNA replication process with features of break-induced replication" by Fani-Marlen Roumelioti et al presents a very significant breakthrough in our understanding of the molecular mechanisms of Alternative Telomere Lengthening (ALT), a pathway responsible for telomere maintenance in approximately 15% of cancers. In particular, the authors used state of the art methods, including telomeric in situ hybridization involving three consecutive staining steps. Using these methods, the authors found the presence of conservatively replicated telomeric DNA in telomerase-negative cancer cells. Another important finding of this study was that depletion of PolD3 and PolD4, two subunits of human polymerase delta that are known to be essential for BIR, reduced the frequency of conservatively replicated telomeric DNA ends and led to shorter telomeres and to the increase of chromosome end-to-end fusions. Together, these two findings confirm two important hypotheses that were proposed based on multiple yeast studies, but were never tested directly in human cells: (i) that BIR is responsible for ALT in humans; and (ii) that BIR in human cells proceeds via conservative DNA synthesis. Overall, the new insights into the mechanisms of ALT resulting from this paper represents a very important development in this field. Because the interest to this topic is very high, I expect that this paper will be frequently cited and will also stimulate further research in various areas including human oncology, DNA repair, replication, and recombination."

We thank the Referee for these very positive comments. We are also excited by the discovery that telomeres in telomerase-negative cells are replicated by conservative DNA replication.

"Specific comments.

1. Fig. 3A and the text on page 7.
Based on the data presented in this figure, the authors proposed that depletion of POLD3 and POLD4 decreases the percent of conservative synthesis at telomeres. Is this reduction statistically significant? What kind of statistics the authors used to confirm this idea? Is the percent of conservative synthesis shown in this figure represent an average from several experiments or the percent based on the results of all experiments combined together?

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The frequency of chromosome end-to-end fusions seem to differ significantly between Ctrl and POLD4? Specifically, the SDs obtained for these two experiments overlap. Also, it remains unclear what kind of statistical methods were used to distinguish between these two groups."

Specific Comments: 1-3, above. Admittedly, in the original version of the manuscript, the statistical analysis was not well described. The revised version of the manuscript describes the statistical analysis according to the instructions provided by the journal. The reported differences are indeed statistically significant and support our conclusions.

Referee #3:

"Roumelioti et al. study the mechanism of recombination mediated telomere lengthening (ALT) in mammalian cells. They address two essential in this field questions. First, is the key break induced replication (BIR) repair pathway protein PolD3 (yeast Pol32) needed for ALT, and second, is the fate of newly synthesized strands similar to a regular replication (semiconservative) or BIR (conservative), as studied in yeast. The authors found out that Pol32 is required for ALT and that synthesis mode reminds the one observed previously in BIR in yeast. Moreover they demonstrate that overexpression of cyclin E stimulates BIR indicating that fork breakage could play a role in ALT. Together, the authors provide many insights into the mechanism of ALT."

We thank the Referee for these positive comments.

"Concerns: A control cell line - non ALT is essential to demonstrate that all the events described here are observed only in ALT positive cells."

We understand that examining a non-ALT cell line would strengthen the study. However, our conclusions are not based on any assumptions about the nature of telomeric DNA replication in non-ALT cells. Specifically, our conclusions relate to ALT cells and we propose that in these cells, BIR plays a key role in telomere maintenance. This is supported by the presence of conservative DNA replication and the dependence of telomere integrity on genes, namely POLD3 and POLD4, that are important for BIR in these cells. Of course, we intend to examine a large panel of non-ALT cells in the future, but we believe that our findings are exciting and novel enough to justify publication at this stage.

"The authors performed a triple FISH staining to distinguish between conservative and semiconservative mode of newly synthesized DNA inheritance. The results implicate that up to few percent of cells have telomeres built exclusively from new or parental strands, it is hard to understand how would it be possible. For any recombination between telomeres (sister or nonsister) to occur there must be some telomeric sequence made during regular replication that engages in

recombination. Thus at least some "semiconservative" part of telomeric sequence must be present on each chromosome end. A comment on this is required."

We envision, as described in the manuscript, that, if fork collapse occurs in the subtelomeric regions, then the entire telomere could be replicated by BIR and display conservative DNA replication. BIR requires sequence homology, but the homology is between sister chromatids, so the subtelomeric regions display the homology needed to initiate BIR. Having said that, the majority of telomeres exhibiting conservative DNA replication also had a segment that was semiconservatively replicated, as quantitated in Fig. 2B (E, entire telomere replicated conservatively; P, part of the telomere replicated conservatively).

"Are the differences presented in Fig. 3A statistically significant? One of the most sensitive ways to test telomere recombination in ALT cells is to examine the presence of byproducts of recombination, C-circles. It would be beneficial to test the presence of C-circle upon PolD3/4 depletion."

Yes, the differences in Fig. 3A and the other figures are statistically significant. We apologize for not having a full statistical analysis in the original version of the manuscript. This has been corrected in the revised version. We had already considered examining for the presence of C-circles. In the end, we decided to focus our resources on the experiments shown in the manuscript, which we believe more directly support our conclusions.

"Minor Comments

Fig 2b, please change "all cnsrv" to "total consrv" as all cnrsv indicate category where whole telo is build from new strands."

We agree that the original terms were confusing. This has been addressed in the revised version of the manuscript.

"It would help to explain earlier in the manuscript why the authors use o/e of cyclin E."

This is now explained earlier in the text, as the Referee suggests.

Accepted 04 October 2016

Thank you for the submission of your revised manuscript. We have now received the referee comments and both referees support its publication, despite the fact that non-ALT cells have not been examined. While the referees agree that these data would strengthen a role for BIR in ALT cells specifically, they also remark that these experiments can be performed in future studies.

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

I have slightly shortened the short summary and bullet points. Please let me know in case you do not agree with the following:

Human cells that rely on ALT to maintain telomere length use break-induced replication, a DNA repair pathway associated with conservative rather than semiconservative DNA replication.

- Telomeres of human ALT cells are replicated conservatively.
- PolD3 and PolD4, two subunits of DNA polymerase delta that function in break-induced replication are needed for the maintenance of telomere length and function in human ALT cells.

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Corresponding Authors: Thanos D. Halazonetis and Sarantis Gagos

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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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was dictated by practice in the field. The assays involve analysis of microscopy images of thousands of chromosome arms stained with various methods as described in the manuscript. We have the power to detect large effects, which we believe would be physiologically relevant.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The microscope slides were scored blindly and after the measurements were completed, the code was broken.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
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.	As mentioned above, the microscope slides were scored blindly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes, we use t tests for interval and ratio data and chi square tests for nominal data.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For certain data, the number of replicates is not sufficient to calculate if they fit a normal distribution. This problem is not specific to our study.
Is there an estimate of variation within each group of data?	Yes, standard errors.
Is the variance similar between the groups that are being statistically compared?	Comparisons of variances also requires a larger number of replicates, which we did not have for all experiments.

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).	Not applicable.
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 U2OS cells used in this study were originally obtained from the ATCC. We have performed a karyotyping and are confident that these are U2OS cells. They were also tested for mycoplasma and were mycoplasma-negative.

* 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.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
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.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
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.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
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.	Not applicable.
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.	Not applicable.

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	Not applicable.
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).	Not applicable.
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).	Not applicable.
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	Not applicable.
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.	Not applicable.

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.	Not applicable.