TERT activates endogenous retroviruses to promote an immunosuppressive tumour microenvironment

Jian Mao, Qian Zhang, Yaxiang Wang, Yang Zhuang, Lu Xu, Xiaohe Ma, Di Guan, Junzhi Zhou, Jiang Liu, Xiaoying Wu, Qian Liang, Miao Wang, and Yu-Sheng Cong
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Review Timeline:

Editor: Achim Breiling

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Cong,

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

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4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an

appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We 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. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that, where applicable, the number "n" for how many independent experiments were performed and the type of replicate (biological or technical), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

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.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

This work proposes a TERT-dependent mechanism leading to an immunesuppressive tumor microenvironment via ERV dsRNA expression and interferon response.

The proposed mechanism is interesting and would reinforce the emerging idea of non-canonical functions of telomerase. However, many arguments rely on correlative results that preclude any firm conclusion and the link between TERT expression in tumors and an immunosuppressive signature might well be indirect.

Moreover, their cellular models to establish causal relationships suffer from an important methodological problem: The effect is observed only when TERT is highly overexpressed, at a level that is even higher than the one observed in tumors. For instance, they use HeLa cells that are tumor cells already overexpressing TERT and the level of overexpression (by WB) is far higher than in the controls. So, it is mandatory to show that ERV activation/interferon response decreases in tumor cells "normally" overexpressing TERT upon TERT knockdown.

An interesting settings would also be to start from normal fibroblasts and to find a way to overexpress TERT in these cells at the level SIMILAR to the one observed in tumor (try a battery of lentiviral vectors with TERT under the control of various promoters to find the "good" one).

Referee #2:

In this study, the authors employ data from The Cancer Genome Atlas to show that expression of telomerase reverse transcriptase (TERT) is correlated with immune-suppressive signatures (classified by Th2, Treg, NK CD56dim cells and MDSCs signatures here). They then show that ectopic expression of both TERT and a catalytically inactive TERT mutant in cell lines induces modest expression of interferon-stimulated genes (ISGs), as well as overexpression of some endogenous retroviruses (ERVs). They show evidence that some of these ERVs are expressed bidirectionally and TERT expression leads to detectable dsRNA staining in U2OS cells. The authors suggest that TERT and SP1 co-bind to these ERVs and induce their transcription leading to a decreased level of 5mC at them, although controls are lacking and effects are small. The authors then generate TERT-/- mice and observe some differences in ERV and chemokine expression. This study is novel and interesting but there are concerns about some of the data as it is presented and concerns with the conclusions drawn. It is not clear how the results of TERT overexpression inducing ISGs relate to the immune-suppresssive signatures in TERT high tumours.

Major:

1. It is interesting that both the TERT WT and catalytically-inactive TERT constructs induce a signature of an innate immune response in U2OS cells by gene set enrichment analyses. However, the fact that WT and mutant induce similar phenotypes should be discussed. Considering these findings, it would also be important to have a negative control construct ectopically expressed (not just transfection of the empty vector) in order to ascertain if the induced innate immune response is related to TERT or if it would occur in the presence of any control construct (GFP for example), especially since effects are small (figure 1cde)?

2. The ISG upregulation observed in figure 1e is modest (2-fold) and would not normally be classified as an ISG response. This suggests that the expression of ISGs may be slightly increased by TERT acting as a transcriptional activator rather than through type I interferon being secreted. This should be explored by doing an IFNb ELISA or bioassay with supernatants from TERT expressing cells.

3. Figure 3a: The MeDIP-qPCR in Figure 3a is interesting. The differences between treatment groups are modest however and it would be important to have positive and negative control PCRs for sites in the genome known to be enriched or not for 5mC.

4. Figure 3c: There are no positive and negative control ChIP-PCRs for sites in the genome known to be bound or not by TERT and SP1. There is no evidence that TERT and SP1 interact. A co-IP would help here. SP1 and TERT are shown to co-bind dsRNAs in the model in figure 5 but there is no data to support this.

5. Figure 4: Effects of TERT knockout on ERV expression and on the CXCL10 response are not that clear and conclusions should be toned down. It may be the case that the small differences in expression of ERVs and chemokines observed reflect differences in a population of cells in blood for example (CD4 T cells or other). It would be important to measure populations of immune (and immune-suppressive) cell populations in WT and KO mice at baseline and upon stimulation with polyIC.

6. With the cancer data, is there a correlation between TERT high tumours and ERV overexpression because ERVs are overexpressed in CD4 T cells, which have infiltrated TERT high tumours? Or are the overexpressed ERVs present in the TERT high tumour cells? It would be useful to clarify too whether better or worse survival is associated with TERT high tumours?

Minor:

1. Figure 1a: The blue and the black dots are too similar in colour to distinguish from each other. It may be clearer to remove the black dots. The terms 'up', 'down' and 'not' are unnecessary as we can see that the red dots are associated with the TERT-high

expressing group. Why are so few dots shown? - I would expect the plot to look more like the one in figure 5a. Since there are so few red dots, it would be useful to name them all.

2. Figure 2e: It is not clear what the positive and negative controls are in the dsRNA IP sequencing experiment? It is known that inverted Alu repeats (Alu-IRs) make duplex RNAs that are associated with MDA5 and are enriched in 5-AZA treated cells and recognized by the J2 dsRNA Ab. There is a background level of Alu-IRs present in WT cell lines too (PMID: 33087935). Were these detected here? What were all the repeat categories detected in WT and TERT-expressing cells and their abundance?

3. Figure 1c: There is a typo in the top gene ontology category. At the moment, it reads 'regulation of defense response to virus to virus'.

4. Page 5: Missing literature citations of papers documenting the association of the immune signatures (Th2, CD56dim NK cells, MDSCs) with immune suppression.

5. Page 7: 'These data suggested that TERT is involved in interferon response by activating the RNA-sensing pathway'. Documenting that TERT ectopic expression leads to expression of several ISGs including CXCL10 does not show that an RNA -sensing pathway is activated. Please revise.

6. Figure 1f: The levels of pTBK1 do not look higher in the TERT-WT samples compared to vector control. Please quantify the Western. Please comment on the observation from this western that the TERT-K626A mutant activates pTBK1 and pIRF3 more than the WT construct.

7. Figure 2c: Upregulation of the stated ERVs is modest (and in 2D). This would be best on a linear (not log2 scale). How were these ERVs selected? Again, a negative control construct ectopically expressed here would be good to see if upregulation of these ERVs is related to expression of TERT. A statistical test should be used to assess differential expression.

8. Figure 3b: I am not sure that this is helpful to include because it does not show that TERT and SP1 co-bind the same sites so these motifs don't add anything.

9. Figure 4: Data would be clearer if normalized expression was shown (WT/control normalized to 1). Figure 4c: Differential expression analyses pipelines should be used here to see what are the most significant changes. The 'row zscore' is not easy to interpret and a comparison with control would make the expression changes clearer.

10. Figure 5a: The annotated labels are not visible. Figure 5 has some redundancy with figure 1 so it may be best to include the most important points of each of these figures into one figure only.

11. Page 10: 'Deletion of Tert leads to decreased ERVs expression and interferon response in mice'. This subtitle should be revised as it could be interpreted that deletion of TERT leads to an interferon response.

Referee #3:

Telomerase activation through TERT induction is well established essential to cellular immortalization and malignant transformation by stabilizing telomere sizes, however, the other roles for telomerase/TERT in carcinogenesis remain to be defined. In the present manuscript, Mao et al show that TERT activates a group of ERVs by interacting with th TF SP1. These ERVs form dsRNAs, sensed by the RIG-1/MDA5-MAVS signalling pathway, thereby triggering interferon signalling in cancer cells. TERT-induced ERVs/interferon signalling then stimulates the expression of chemokines, and subsequently induces suppressed T cell infiltration with increased percentage of CD4+ and FOXP3+ cells. Thus, the authors conclude that TERT-mediated ERVs/interferon signalling contributes to immune suppression in tumours. The findings have both biological and clinical implications. The study was well performed.

Points:
1. The manuscript is too long.

2. The abstract shouldn't include references.

Responses to the Reviewer comments:

Referee #1:

This work proposes a TERT-dependent mechanism leading to an immunesuppressive tumor microenvironment via ERV dsRNA expression and interferon response.

The proposed mechanism is interesting and would reinforce the emerging idea of non-canonical functions of telomerase. However, many arguments rely on correlative results that preclude any firm conclusion and the link between TERT expression in tumors and an immunosuppressive signature might well be indirect.

Moreover, their cellular models to establish causal relationships suffer from an important methodological problem: The effect is observed only when TERT is highly overexpressed, at a level that is even higher than the one observed in tumors. For instance, they use HeLa cells that are tumor cells already overexpressing TERT and the level of overexpression (by WB) is far higher than in the controls. So, it is mandatory to show that ERV activation/interferon response decreases in tumor cells "normally" overexpressing TERT upon TERT knockdown.

Response: We thank the reviewer for raising this point. In the revised manuscript, we have ectopically expressed TERT at the levels comparable to that of HeLa cells in telomerase negative cancer cells (U2OS) or fibroblasts (WI38), and found that interferon response and TA-ERVs expression were also activated. Furthermore, we have knocked down TERT by siRNAs in HeLa and HCT116 cells and found that the expression of interferon-related genes and TA-ERVs was downregulated (Figs 1G and 2E), the phosphorylation of TBK1 and IRF3 was reduced (Fig 1H), and level of CXCL10 in culture supernatant significantly decreased (Fig 1I), in TERT knocked down cells compared with the control cells. Collectively, these results support that TERT regulates TA-ERVs expression and interferon response.

An interesting setting would also be to start from normal fibroblasts and to find a way

to overexpress TERT in these cells at the level SIMILAR to the one observed in tumor (try a battery of lentiviral vectors with TERT under the control of various promoters to find the "good" one).

Response: We thank the reviewer for this constructive suggestion. In the revised manuscript, we have used Dox-inducible system to control the expression of TERT in human fibroblasts WI38 cells at the level comparable to that of HeLa cells, GFP was used as negative control. We obtained consistent results that TERT triggers interferon response and activates TA-ERVs expression (Figs EV1D-G), which was not the effects due to overexpression of TERT.

Referee #2:

In this study, the authors employ data from The Cancer Genome Atlas to show that expression of telomerase reverse transcriptase (TERT) is correlated with immune-suppressive signatures (classified by Th2, Treg, NK CD56dim cells and MDSCs signatures here). They then show that ectopic expression of both TERT and a catalytically inactive TERT mutant in cell lines induces modest expression of interferon-stimulated genes (ISGs), as well as overexpression of some endogenous retroviruses (ERVs). They show evidence that some of these ERVs are expressed bidirectionally and TERT expression leads to detectable dsRNA staining in U2OS cells. The authors suggest that TERT and SP1 co-bind to these ERVs and induce their transcription leading to a decreased level of 5mC at them, although controls are lacking and effects are small. The authors then generate TERT-/- mice and observe some differences in ERV and chemokine expression. This study is novel and interesting but there are concerns about some of the data as it is presented and concerns with the conclusions drawn. It is not clear how the results of TERT overexpression inducing ISGs relate to the immune-suppresssive signatures in TERT high tumours.

Major:

1. It is interesting that both the TERT WT and catalytically-inactive TERT constructs induce a signature of an innate immune response in U2OS cells by gene set enrichment analyses. However, the fact that WT and mutant induce similar phenotypes should be discussed. Considering these findings, it would also be important to have a negative control construct ectopically expressed (not just transfection of the empty vector) in order to ascertain if the induced innate immune response is related to TERT or if it would occur in the presence of any control construct (GFP for example), especially since effects are small (figure 1cde)?

Response: We thank the reviewer for this suggestion. TERT-K626A used in this study is a mutant deficient in telomerase catalytic activity (it cannot synthesize telomere), but it maintains the ability to regulate genes expression like TERT-WT. We have previously reported that both TERT-WT and TERT-K626A regulate *VEGF* and *MMP*s expression independent of telomerase catalytic activity (Ding *et al.*, 2013, Liu *et al.*, 2016). In this study, we found that TERT activates TA-ERVs expression independent of telomerase catalytic activity, and both TERT-WT and TERT-K626A induced similar phenotypes.

To ascertain the interferon response and TA-ERVs activation are related to TERT specifically, in the revised manuscript, we used Dox-inducible system to control the TERT expression at the levels comparable to that of HeLa in U2OS and WI38 cells, and GFP was used as negative control (Fig EV1D). We obtained consistent results to the previous experiments using empty vector as negative control. Upon induction of TERT expression, we detected the upregulation of interferon-related genes and TA-ERVs (Figs 2F and EV1E), activation of TBK1 and IRF3 (Figs 2G and EV1F), and increased IFNβ and CXCL10 in culture supernatant (Figs 2H and EV1G). These results support that TERT triggers interferon response and activates TA-ERVs expression.

2. The ISG upregulation observed in figure 1e is modest (2-fold) and would not

normally be classified as an ISG response. This suggests that the expression of ISGs may be slightly increased by TERT acting as a transcriptional activator rather than through type I interferon being secreted. This should be explored by doing an IFNb ELISA or bioassay with supernatants from TERT expressing cells.

Response: We thank the reviewer for this suggestion. As recommended by the reviewer, in the revised manuscript, we have performed the ELISA for IFNβ with the cell culture supernatants from vector (or GFP), TERT-WT and K626A expressing cells (including U2OS, HeLa, and WI38 cells), the results showed that levels of IFNβ increased in cell culture supernatant upon TERT expression (Figs 1F, 2H, and EV1G). These results indicate that TERT is involved in type I interferon response.

3. Figure 3a: The MeDIP-qPCR in Figure 3a is interesting. The differences between treatment groups are modest however and it would be important to have positive and negative control PCRs for sites in the genome known to be enriched or not for 5mC.

Response: We thank the reviewer for this valuable suggestion. In the revised manuscript, we have included commercial primer sets recognizing exon 8 of the human *ZC3H13* gene (Human Positive Control Primer Set ZC3H13, Active Motif, 71009) or a gene desert on chromosome 12 (Human Negative Control Primer Set 1, Active Motif, 71001) as positive and negative controls, respectively, in the MeDIP-qPCR experiments. The results confirm that the methylation levels of MLT1O-int, LTR69, LTR38-int, MER88, MER92C, and MLT1G1-int indeed decreased in HeLa cells ectopically expressing TERT-WT or TERT-K626A (Fig 3D).

4. Figure 3c: There are no positive and negative control ChIP-PCRs for sites in the genome known to be bound or not by TERT and SP1. There is no evidence that TERT and SP1 interact. A co-IP would help here. SP1 and TERT are shown to co-bind dsRNAs in the model in figure 5 but there is no data to support this.

Response: We thank the reviewer for pointing this out. In the revised manuscript, we have included the *GAPDH* promoter as a negative control, and the *VEGF* promoter as positive control (Fig 3E). Actually, we have previously reported that TERT activates

VEGF expression via its interaction with the Sp1 transcription factor (Liu *et al*. hTERT promotes tumor angiogenesis by activating VEGF via interactions with the Sp1 transcription factor. *Nucleic Acids Res* 44: 8693-8703, 2016), in which we have demonstrated the interaction between TERT and Sp1 by co-IP and *in vitro* pull-down assay. In the model of Fig 5H, TERT and Sp1 co-bind to genomic ERVs sites to activate ERVs expression. The dsRNAs are transcription products of these ERVs via bidirectional transcription (blue and yellow arrows indicating two transcription directions, respectively). Thus, TERT and Sp1 bind to genomic ERVs sites but not to dsRNAs. Indeed, we realized that the model looks confusing, therefore we have improved it in the revised manuscript (Fig 5H).

5. Figure 4: Effects of TERT knockout on ERV expression and on the CXCL10 response are not that clear and conclusions should be toned down. It may be the case that the small differences in expression of ERVs and chemokines observed reflect differences in a population of cells in blood for example (CD4 T cells or other). It would be important to measure populations of immune (and immune-suppressive) cell populations in WT and KO mice at baseline and upon stimulation with polyIC.

Response: We thank the reviewer for this suggestion. In the revised manuscript, in addition to whole blood, we also measured MuERVs expression in livers of WT and G1 $Tert^{-/-}$ mice, the results confirmed the downregulation of MuERVs in livers of G1 $Tert^{-/-}$ mice (Fig EV3B). As suggested by the reviewer, we have measured the populations of CD4$^+$/FOXP3$^+$ cells in blood of WT and G1 $Tert^{-/-}$ mice at baseline and upon stimulation with poly(I:C) by flow cytometry analysis. The results showed that the population of CD4$^+$ and CD4$^+$FOXP3$^+$ cells increased under poly(I:C) treatment in both WT and G1 $Tert^{-/-}$ mice, but there is no significant difference observed in population of CD4$^+$ and CD4$^+$FOXP3$^+$ cells between WT and G1 $Tert^{-/-}$ mice at baseline or upon stimulation with poly(I:C) (Figs EV3C and D), confirming that the differences observed in expression of ERVs and cytokine/chemokine are due to the activation of ERVs by TERT but not differences in population of immune cells in blood.

6. With the cancer data, is there a correlation between TERT high tumours and ERV overexpression because ERVs are overexpressed in CD4 T cells, which have infiltrated TERT high tumours? Or are the overexpressed ERVs present in the TERT high tumour cells? It would be useful to clarify too whether better or worse survival is associated with TERT high tumours?

Response: This is an interesting question. We thank the reviewer for pointing this out. Our data obtained from cells and mice studies, including TERT ectopic expression and knockdown, G1 *Tert*$^{-/-}$ compared with WT mice, MeDIP-qPCR and ChIP-qPCR analyses, have demonstrated that TERT regulates the expression of TA-ERVs and triggers interferon response. Therefore, TERT plays a major role in upregulating ERVs expression in TERT high tumours, and it is unlikely that ERVs upregulation by TERT was due to CD4 T cell population.

For the survival associated with TERT, as the reviewer mentioned that there have been many reports revealed that TERT high tumours are associated with worse survival. For example, elevated TERT mRNA expression strongly correlates with reduced disease-specific survival in urothelial cancer patients (Borah *et al.*, 2015). Colorectal cancer (CRC) patients with high TERT levels show a significantly worse survival than those with low TERT levels and suffer a higher risk of disease recurrence and death in stage II CRC (Bertorelle *et al.*, 2013). We have discussed this in discussion section.

Minor:

1. Figure 1a: The blue and the black dots are too similar in colour to distinguish from each other. It may be clearer to remove the black dots. The terms 'up', 'down' and 'not' are unnecessary as we can see that the red dots are associated with the TERT-high expressing group. Why are so few dots shown? - I would expect the plot to look more like the one in figure 5a. Since there are so few red dots, it would be useful to name them all.

Response: We thank the reviewer for this helpful suggestion and revised the manuscript accordingly. In Figs 1A and 1B, we quantified the relative infiltration of 25 immune cell types in the tumor microenvironment for 9,264 tumor samples from TCGA by ssGSEA. Therefore, Figs 1A (volcano plot) and 1B (heat map) show the 25 immune cell types. To make this figure clearer, we have removed the black dots and the terms "up", "down", "not", and named all the dots (Fig 1A). Fig 5A is the ssGSEA in TCGA for "CP: Canonical pathways" downloaded from Molecular Signatures Database on GSEA website, which contains a large number of gene sets from several pathway databases, such as KEGG, REACTOME, etc. Therefore, Fig 5A exhibits a lot of dots, each dot represents a signal pathway.

2. Figure 2e: It is not clear what the positive and negative controls are in the dsRNA IP sequencing experiment? It is known that inverted Alu repeats (Alu-IRs) make duplex RNAs that are associated with MDA5 and are enriched in 5-AZA treated cells and recognized by the J2 dsRNA Ab. There is a background level of Alu-IRs present in WT cell lines too (PMID: 33087935). Were these detected here? What were all the repeat categories detected in WT and TERT-expressing cells and their abundance?

Response: We thank the reviewer for this helpful suggestion. In the revised manuscript, we have included the positive (AluYb9) and negative (tRNA-Thr-ACG) controls for the dsRIP-seq (Fig 3A). Besides ERVs, other repeat categories, including LINE (such as L1 family) and SINE (such as Alu family), were also detected at considerable levels in our dsRIP-seq. We have revised the Fig 3A accordingly.

3. Figure 1c: There is a typo in the top gene ontology category. At the moment, it reads 'regulation of defense response to virus to virus'.

Response: Thanks for the correction. We have corrected it to "regulation of defense response to virus by virus" in Fig EV1B.

4. Page 5: Missing literature citations of papers documenting the association of the immune signatures (Th2, CD56dim NK cells, MDSCs) with immune suppression.

Response: Thanks for pointing this out. We have added a suitable reference for this statement: Jia Q, *et al*. (2018) Local mutational diversity drives intratumoral immune heterogeneity in non-small cell lung cancer. *Nat Commun* 9: 5361 (Line 85-86).

5. Page 7: 'These data suggested that TERT is involved in interferon response by activating the RNA-sensing pathway'.
Documenting that TERT ectopic expression leads to expression of several ISGs including CXCL10 does not show that an RNA -sensing pathway is activated. Please revise.

Response: We thank the reviewer for the correction. We have revised it to "These data suggested that TERT is involved in interferon response" (Line 118-119).

6. Figure 1f: The levels of pTBK1 do not look higher in the TERT-WT samples compared to vector control. Please quantify the Western. Please comment on the observation from this western that the TERT-K626A mutant activates pTBK1 and pIRF3 more than the WT construct.

Response: We thank the reviewer for this suggestion. We have quantified the western blot by ImageJ, and normalized the intensity of pTBK1 and pIRF3 by GAPDH, and set controls (Vector/GFP/siNC) as 1 in Figs 1E, 1H, 2G and EV1F. We also noticed that TERT-K626A activate interferon response and TA-ERVs expression more significant than TERT-WT. It is possible that the TERT-K626A mutant has higher transcriptional efficiency, as it won't be taken up by telomeres.

7. Figure 2c: Upregulation of the stated ERVs is modest (and in 2D). This would be best on a linear (not log2 scale). How were these ERVs selected? Again, a negative control construct ectopically expressed here would be good to see if upregulation of these ERVs is related to expression of TERT. A statistical test should be used to assess differential expression.

Response: We thank the reviewer for this suggestion. We have changed the log2 fold change to a linear fold change in Fig 2C. For the selection of representative TA-ERVs,

we firstly screened the top upregulated ERVs both in TERT-WT and TERT-K626A (Fig 2C), then verified these ERVs expression by RT-qPCR, finally selected the significantly upregulated ERVs as representative TA-ERVs (Fig 2D). Data of RNA-seq were served as a preliminary screening for TA-ERVs, we have verified the expression of these ERVs using RT-qPCR. Furthermore, we have used several experimental systems in cells and mice studies, including TERT ectopic expression and knockdown, G1 $Tert^{-/-}$ compared with WT mice, MeDIP-qPCR and ChIP-qPCR analyses, to confirm that the upregulation of these ERVs is indeed related to TERT.

8. Figure 3b: I am not sure that this is helpful to include because it does not show that TERT and SP1 co-bind the same sites so these motifs don't add anything.

Response: Thanks for this suggestion. We have moved the motif analysis to Fig EV2B. Based on the motif analysis of TA-ERVs, we found that the sequences of TA-ERVs contained Sp1 motif. As Sp1 has been reported to bind to ERVs and protect them from methylation (Manghera *et al.*, 2013), and TERT can interact with Sp1 (our previous study, Liu *et al.*, 2016), we speculated that TERT may activate ERVs via interaction with Sp1. Then we performed the ChIP-qPCR for TERT and Sp1, and found that TERT and Sp1 are both enriched at the same genomic sites of TA-ERVs. These results suggest that TERT and Sp1 co-bind the genomic TA-ERVs sites to activate their expression. Therefore, motif analysis (Fig EV2B) may be helpful for understanding the regulation of ERVs by the interaction of TERT and Sp1.

9. Figure 4: Data would be clearer if normalized expression was shown (WT/control normalized to 1). Figure 4c: Differential expression analyses pipelines should be used here to see what are the most significant changes. The 'row zscore' is not easy to interpret and a comparison with control would make the expression changes clearer.

Response: We thank the reviewer for this suggestion. We have normalized the expression of WT/control to 1 for the RT-qPCR in Figs 4A, 4B and EV3B. For Fig 4C, we performed differential expression and GO analyses between WT and G1 $Tert^{-/-}$ mice both under control (saline) and ENU treatment and found significant

upregulation for GO terms associated with viral defence and interferon response in WT mice, especially under ENU treatment, compared to G1 $Tert^{-/-}$ mice (Fig 4C). Indeed, the "row zscore" is not easy to interpret, we have revised this figure to a comparison (log2foldchange) between ENU and control for WT/G1 $Tert^{-/-}$ mice and the expression of interferon-related genes was significantly upregulated by ENU in WT mice but not in G1 $Tert^{-/-}$ mice (Fig 4D).

10. Figure 5a: The annotated labels are not visible. Figure 5 has some redundancy with figure 1 so it may be best to include the most important points of each of these figures into one figure only.

Response: Thank you for pointing this issue. We have revised this figure to make the labels clearer (Fig 5A). As mentioned above, Figs 1A and 5A are data for different purposes and illustrated different issues. Therefore, we think it is suitable to be shown as two figures.

11. Page 10: 'Deletion of Tert leads to decreased ERVs expression and interferon response in mice'. This subtitle should be revised as it could be interpreted that deletion of TERT leads to an interferon response.

Response: We thank the reviewer for correction. We have revised it to "Deletion of *Tert* leads to decreased ERVs expression and compromised interferon response in mice" (Line 195-196).

Referee #3:

Telomerase activation through TERT induction is well established essential to cellular immortalization and malignant transformation by stabilizing telomere sizes, however, the other roles for telomerase/TERT in carcinogenesis remain to be defined. In the present manuscript, Mao et al show that TERT activates a group of ERVs by

interacting with th TF SP1. These ERVs form dsRNAs, sensed by the RIG-1/MDA5-MAVS signalling pathway, thereby triggering interferon signalling in cancer cells. TERT-induced ERVs/interferon signalling then stimulates the expression of chemokines, and subsequently induces suppressed T cell infiltration with increased percentage of CD4+ and FOXP3+ cells. Thus, the authors conclude that TERT-mediated ERVs/interferon signalling contributes to immune suppression in tumours. The findings have both biological and clinical implications. The study was well performed.

Points:

1. The manuscript is too long.

Response: We thank the reviewer for pointing this out. We have revised the manuscript to make the words and format meet the requirements of the journal.

2. The abstract shouldn't include references.

Response: Thank you for the correction. We have removed the references in abstract.

References

Bertorelle R, Briarava M, Rampazzo E, Biasini L, Agostini M, Maretto I, Lonardi S, Friso ML, Mescoli C, Zagonel V, Nitti D, De Rossi A, Pucciarelli S (2013) Telomerase is an independent prognostic marker of overall survival in patients with colorectal cancer. *Br J Cancer* 108: 278-284

Borah S, Xi L, Zaug AJ, Powell NM, Dancik GM, Cohen SB, Costello JC, Theodorescu D, Cech TR (2015) Cancer. TERT promoter mutations and telomerase reactivation in urothelial cancer. *Science* 347: 1006-1010

Ding D, Xi P, Zhou J, Wang M, Cong Y-S (2013) Human telomerase reverse transcriptase regulates MMP expression independently of telomerase activity via NF-κB-dependent

transcription. *FASEB J* 27: 4375-4383

Liu N, Ding D, Hao W, Yang F, Wu X, Wang M, Xu X, Ju Z, Liu J-P, Song Z, Shay JW, Guo Y,

    Cong Y-S (2016) hTERT promotes tumor angiogenesis by activating VEGF via

    interactions with the Sp1 transcription factor. *Nucleic Acids Res* 44: 8693-8703

Manghera M, Douville RN (2013) Endogenous retrovirus-K promoter: a landing strip for

    inflammatory transcription factors? *Retrovirology* 10: 16

Dear Prof. Cong,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the report from one of the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referee now fully supports the publication. Referee #1 had agreed to look into this again but has so far not submitted his/her report and is completely unresponsive to our reminders. However, going through your p-b-p-response and the revised manuscript I consider the points of referee #1 (and also the minor requests by original referee #3) as adequately addressed. Referee #2 has a final request we ask you to address in a final revised manuscript.

Moreover, I have these editorial requests I also ask you to address:

- I would suggest this modified title:
TERT activates endogenous retroviruses to promote an immunosuppressive tumour microenvironment.

- Please provide the abstract written in present tense.

- We plan to publish your manuscript in the Report format (as you also indicated in the submission system). For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- As they are significantly cropped, please provide the source data for the Western blots shown in the manuscript (including the EV and Appendix figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the final 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 (main and EV figures).

- Please also show the Western blots as unmodified as possible, resembling the source data. Presently, several blots shown are overcontrasted (e.g. those shown in Fig. EV1D and EV1F). Please show all blots in one figure panel with same contrast and brightness, resembling closely the original data.

- I would suggest moving all the EV tables into an Appendix file, as these do not contain main data. Please upload these in one pdf file named 'Appendix' with page numbers and use 'Appendix Table S#' to name the tables. Please use this also as callout in the manuscript text and update all callouts. Please make sure these tables are called out (presently, it seems Table EV4 is not called out). Please add a table of contents to the Appendix legends including page numbers and also add titles and legends to the tables. Finally, please remove the tables from the main manuscript file.

- In Fig. EV2A for some samples you indicate n=2, but nevertheless show error bars. Please either show more than 2 samples or show the two datapoints separately, removing the error bars for those samples with n=2.

- Please format the references according to our journal style (we need et al. for publications with more than 10 authors). See also:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels)

that can be used as a visual synopsis on our website.

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

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #2:

The authors have answered all of my comments and this ms is now suitable for publication. Before publication, the abbreviated immune subtypes in Figure 1a should be written in full in the legend (aDC, Tgd etc.).

Responses to editor and reviewer comments:

- I would suggest this modified title:

TERT activates endogenous retroviruses to promote an immunosuppressive tumour microenvironment.

Response: Thank you for the suggestion. We have modified the title as you suggested.

- Please provide the abstract written in present tense.

Response: Thank you for the correction. We have revised the abstract in present tense.

- We plan to publish your manuscript in the Report format (as you also indicated in the submission system). For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

Response: We have revised the manuscript according to the Report format.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

Response: We have indicated "ns" for those testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these

in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

Response: We have revised the scale bars in consistent style and thickness for the microscopic images.

- As they are significantly cropped, please provide the source data for the Western blots shown in the manuscript (including the EV and Appendix figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the final 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 (main and EV figures).

Response: We have provided source data for all the western blots shown in our manuscript.

- Please also show the Western blots as unmodified as possible, resembling the source data. Presently, several blots shown are overcontrasted (e.g. those shown in Fig. EV1D and EV1F). Please show all blots in one figure panel with same contrast and brightness, resembling closely the original data.

Response: Thank you for pointing this out. We have shown the western blots resembling the source data and re-quantified the blots of Fig EV1F.

- I would suggest moving all the EV tables into an Appendix file, as these do not contain main data. Please upload these in one pdf file named 'Appendix' with page numbers and use 'Appendix Table S#' to name the tables. Please use this also as callout in the manuscript text and update all callouts. Please make sure these tables are called out (presently, it seems Table EV4 is not called out). Please add a table of contents to the Appendix legends including page numbers and also add titles and legends to the tables. Finally, please remove the tables from the main manuscript file.

Response: Thank you for the suggestion. We have moved all the EV tables into an

- In Fig. EV2A for some samples you indicate n=2, but nevertheless show error bars. Please either show more than 2 samples or show the two datapoints separately, removing the error bars for those samples with n=2.

- Please format the references according to our journal style (we need et al. for publications with more than 10 authors). See also:

http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:

- a short, two-sentence summary of the manuscript (not more than 35 words).

- two to four short bullet points highlighting the key findings of your study.

- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

Referee #2:

The authors have answered all of my comments and this ms is now suitable for publication. Before publication, the abbreviated immune subtypes in Figure 1a should be written in full in the legend (aDC, Tgd etc.).

Response: We thank the reviewer for the kind consideration of our manuscript and suggestions. We have revised the legend of Fig 1 with the full names for abbreviations.

Prof. Yu-Sheng Cong
Hangzhou Normal University
Key Laboratory of Aging and Cancer Biology of Zhejiang Province
2318 Yuhangtang Rd
Zhejiang 311121
China

Dear Prof. Cong,

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

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	At least three biological replicates were performed in our experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded in this study.
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.	Controls were always setup at the same time as treatment groups. Processing of animals occurred randomized and blinded.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were randomly selected in the cages before allocating them to treatment groups or control groups.
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.	Controls were always setup at the same time as treatment groups. Except for experimental operations, control and treatment groups are maintained under same conditions.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The authors who did the experiments were blinded to group allocation during data collection and analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests used in each figure are described in the figure legends (and Materials and Methods section).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes (reported in every figure legend).

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-tum

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

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http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes, error bars in our data were presented as mean±SEM/SD.
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).	Catalog number and manifacturer are provided in Materials and Methods section for each antibody.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Confirmed.

* 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.	Done (provided in Materials and Methods section).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Done (provided in Materials and Methods section).
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.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Done: RNA-seq data are available in the GEO database with GSE169715 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE169715).
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. 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

22. 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