nature research

Peer Review Information

Journal: Nature Cell Biology
Manuscript Title: Maintenance of R-loop structures by phosphorylated hTERT preserves genome integrity
Corresponding author name(s): Professor Kenkichi Masutomi

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:

*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Professor Masutomi,

Your manuscript, "Maintenance of R-loop structures by phosphorylated hTERT preserves genome integrity", has now been seen by 3 referees, who are experts in telomerase and cancer (referee 1); R-loops and phase separation (referee 2); and R-loops (referee 3). As you will see from their comments (attached below) they find this work of potential interest, but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further.

In particular, it would be essential to:

A: Perform further experiments to establish the specificity of the p-hTERT antibody (for IF and IP) (reviewer#1 pts 1,6, reviewer#2 pts 11-12) and test both p-Htert antibodies in the same cell lines (reviewer#1 pt2)

B: Conduct further experiments to test the proposed model (that p-hTERT specifically associated with

TERRA to carry out RdRP function and suppress R-loops), as per reviewers recommendations (reviewer#1 pt 2, reviewer#2 pt 13-16, reviewer#3 paragraph 2, 4)

C: Test whether hTERT and p-TERT colocalise in cells (Referee#2 pt 3,9,10 Reviewer#1 Ept2,)

D: All other referee concerns pertaining to strengthening existing data, providing controls(Reviewer#1 pt1, reviewer#2 pt6,8), methodological details, clarifications and textual changes, should also be addressed.

E: Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and www.nature.com/nature/authors/).

- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.

- provide the completed Editorial Policy Checklist (found here https://www.nature.com/authors/policies/Policy.pdf), and Reporting Summary (found here https://www.nature.com/authors/policies/ReportingSummary.pdf). This is essential for reconsideration of the manuscript and these documents will be available to editors and referees in the event of peer review. For more information see http://www.nature.com/authors/policies/availability.html or contact me.

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achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

[Redacted]

*This url links to your confidential home page and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We would like to receive a revised submission within six months. We would be happy to consider a revision even after this timeframe, however if the resubmission deadline is missed and the paper is eventually published, the submission date will be the date when the revised manuscript was received.

We hope that you will find our referees' comments, and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss.

Best wishes,

Sabrya Carim

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Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
A. Summary of results
• ALT (and telomerase-positive [TEL]) cells contain a protein recognised by an anti-phTERT antibody; the quantity is increased in mitosis and reduced by treatment of cells with a CDK1 inhibitor (CDK1i)
• Primers specific for hTERT exons 15-16 detected a band in three ALT cell lines at a level approximately one-third of that in HeLa cells (TEL)
• Protein pulled down by p-hTERT antibody did not contain hTERC RNA, had no telomerase activity (TA), and exhibited RdRP activity; this was decreased by prior treatment with siTERT and CDK1i
• p-hTERT localizes in nuclear speckles (NS); this was disrupted in cells by 1,6-HD, a disruptor of

hydrophobic interactions, and by cell lysis in high salt conditions
• NS-enriched cell fractions were found to be depleted of hTERC and to contain protein that was pulled down by anti-p-hTERT antibody, detected by hTERT immunoblotting, and exhibited RdRP activity
• RIP-Seq analysis showed that hTERT associated with telomere repeat-containing noncoding RNAs (TERRA) and that siTERT-treated cells had higher levels of TERRA
• hTERT immune complex (IC) had RdRP activity with TERRA RNA (this appeared to be preferential over RNAs without TERRA sequences); bound to ds-RNAs (detected by J2 antibody); and degraded telomeric repeat RNA:DNA hybrids
• The authors therefore concluded that the RdRP activity in hTERT IC disrupts telomeric R-loops by synthesizing dsTERRA
• CRISPR-induced disruption of hTERT expression in an ALT cell line (U2-OS) decreased proliferation in vitro and tumor growth in immunosuppressed animals and resulted in increased vulnerability to DNA damage (ionizing radiation or DNA damage)
• A genome-wide CRISPR-knockout screen showed that the hTERT-CRISPR cells were dependent on BRCA1, BRCA2 and other FANC genes, and double knockdown of hTERT and FANC/BRCA genes showed increased accumulation of R-loops detected by S9.6 antibody; the hTERT and FANC/BRCA pathways did not appear to be epistatic

B. Originality and significance
It appears that this is essentially the only lab that has published on the subject of hTERT-associated RdRP activity since the senior author published the first paper during his post-doc in the Hahn laboratory. This manuscript contains many novel findings, and is a conceptual advance on the previous papers on this subject. If confirmed, this is an important finding about a noncanonical role of hTERT or a protein with which it co-immunoprecipitates.

C. Data and methodology
The quality of the data appears to be high, and the data are well-presented

D. Statistics
N/A

E. Conclusions
1.The authors mostly assume that hTERT possesses RdRP activity without discussing the possibility that it may be a protein that associates with hTERT.

2. I think there is a major problem with the conclusion that the RdRP activity is responsible for R-loop unwinding at the telomere given that it appears to located in nuclear speckles. The authors should clarify this.

F. Suggested improvements

1. Regarding the pTERT IF results (e.g., Figures 1a, b, c, e), visualizing endogenous levels of TERT has been notoriously difficult. It would be desirable to provide a negative control, e.g., a cell in which TERT has been knocked out. Given the difficulties in achieving this with CRISPR, maybe the U2-OS CRISPR clones could be treated with TERT siRNA to produce a negative control.

2. Could the authors comment on the possibility that alternative splicing is involved. The dominant TERT band and the dominant pTERT bands on WB in figures 1f and g appear to be the same size. Is it

possible that these are a catalytically inactive spliced form of the protein? The paper that first described TERT alternative splicing showed that an ALT cell line expressed alternatively spliced TERT (Kilian A, et al. Hum Mol Genet. 1997;6(12):2011-9).

3. Figure 2 – Has the RdRP assay been validated in any other lab? I note that the 2009 Nature paper has been quite extensively cited (>400 times) but I was unable to easily find a paper that reproduced the assay. Could the authors please comment on this?

4. Given that hTERC is not involved, if hTERT is responsible for RdRP activity there is presumably no requirement for holoenzyme assembly so it may therefore be possible to produce highly-purified TERT protein for this assay in a non-mammalian expression system. This would also facilitate the testing of various splice forms and separation-of-function TERT mutants.

5. hTERT-CRISPR clones: please indicate the location of the 16 bp deletion. What are the predicted consequences of the frameshift? Is the resulting hTERT protein full-length?

6. Minor: the telomerase status of the Capan-1 and TOV-21G cells should be stated

7. Minor: On p.9, the sentence commencing "Combination of both treatments …" is redundant.

G. References
Appropriate
The authors may wish to comment on the previously published data on gene dependency in ALT cells (e.g., Lu and Pickett, Open Biol 12: 220011)

H. Clarity
The paper is very well-written with relatively few typos. Only minor editing is required to make the English more idiomatic.

Reviewer #2:
Remarks to the Author:
In this manuscript, the authors claimed that they detected an hTERT-dependent but hTERC-independent RdRP activity in ALT+ cells. They show that phosphorylated hTERT does not bind hTERC and localize to APBs/telomeres, but is enriched in nuclear speckles and exhibits RdRP activity. Furthermore, they suggest that hTERT binds TERRA and suppresses R-loops through its RdRP activity. Though a CRISPR screen, they found that loss of BRCA1/2 and FA genes is synthetic lethal with loss of hTERT. They proposed that hTERT can function as a suppressor of R-loops through its RdRP activity independently of telomerase, which is important for the survival of ALT+ cells and formation of ALT+ tumors. Although some of the results of this study are interesting, many experiments are not adequately controlled and carefully interpreted. The conclusions of this study are not yet convincing.

1. In Figure 1a-1c, the specificity of the p-hTERT antibody is still not fully established. Phospho-specific antibodies are known to have a high tendency to cross-react. The authors must prove that the p-hTERT detects hTERT but not other phosphorylated proteins. The CDK1 inhibitor experiment only shows the phospho site is CDK1 dependent, but it does not show the protein is hTERT. The specificity control in Fig. 1i was only done with western but not IF, and only with U2OS. This is a major issue for

the paper.

2. The two p-hTERT antibodies were not always tested in the same cell lines in different panels. This makes it difficult to judge whether the two antibodies are completely consistent. If the two antibodies give different results, reader need to know and be cautions with interpretations.

3. Do hTERT and p-TERT colocalized in cells? This is a simple experiment that must be done. It is surprising that the authors have not done it for the manuscript. If hTERT and p-hTERT do not colocalize well, reader should know, and it should be explained.

4. In Figure 1d, both VA-13 and Saos-2 cells express both hTERT and hTERC. Why are these cell lines negative for telomerase activity? The relative expression of hTERT and hTERC in different cell lines may not be meaningful for predicting telomerase activity. Fig. 1d can be misleading.

5. In Fig. 1f, it is surprising that hTERT protein was only detected in nocodazole arrested mitotic cells. Many previous studies have detected hTERT in G1 and S phase cells. Whether the 10E9-2 antibody can detect unphosphorylated hTERT is questionable.

6. Fig. 2a and 2c are not convincing because we don't know how much hTERT protein was used in each sample. Because the expression levels of hTERT are different in different cell lines, it is hard to compared immunoprecipitated hTERT complexes directly. Again, the specificities of TpMab-3 and 10E2-3 for IP need to be established. One cannot assume these antibodies are specific for intended targets in immunoprecipitation without evidence.

7. The interpretation of Fig. 2b is also problematic. The epitope detected by the TpMab-3 antibody may be incompatible with hTERC binding, but it does not necessarily mean that phosphorylated hTERT cannot bind hTERC. The phospho site is not the only determinant in the TpMab-3 epitope. The conclusion needs to be confirmed by other means.

8. The data in Fig. 2d-2f are not meaningful because we don't how much hTERT was used in each sample. Even if RdRP activities were detected in ALT cell lines, it is difficult to judge how robust these activities are. It is impossible to conclude whether RdRP activity is the same or different among ALT+ and telomerase+ cell lines.

9. Given that p-hTERT is localized to nuclear speckles where many phospho-SR proteins are enriched, it is very important to demonstrate the p-hTERT antibody does not cross-react with phospho-SR proteins. This is important for the interpretations of many experiments.

10. Fig. S3 suggests that p-hTERT undergoes LLPS in both ALT+ and telomerase+ cells. It is inappropriate to conclude that p-hTERT is specifically enriched in nuclear speckles and promotes RdRP activity in nuclear speckles in ALT+ cells (page 9). This is a problem for the model of this paper.

11. In Fig. 4a-4b, the p-hTERT antibody (instead of the hTERT antibody) should be used for IP. If p-hTERT does not associate with APBs/telomeres, it may not bind TERRA as un-phosphorylated hTERT. This is a major issue for the model.

12. Again, in Fig. 4c-4f, the p-hTERT antibody should be used for IP. All the data DO NOT support the model that p-hTERT specifically associates with TERRA to carry out RdRP function and suppress R-

loops. This is a major issue for the model.

13. The model gets very confusing because it is not clear where p-hTERT binds TERRA and suppresses R-loops. If p-hTERT does not localize to APBs/telomeres, does it suppress R-loops in nuclear speckles? Why is TERRA involved in R-loop regulation in nuclear speckles? TERRA is known to be enriched at telomeres in ALT+ and ALT- cells.

14. hTERT is known to have telomerase independent functions. The interpretation of Fig. 5 is difficult. The CRISPR/Cas9 editing of hTERT was only done in U2OS, and it is not clear whether the effects are specific to ALT+ cells. It is also unclear whether the effects are related to R-loops or telomeres. These experiments do not directly support the model of this paper.

15. The data in Fig. 6 is difficult to interpret because it is inappropriate to use hTERT-CRISPR cells and RdRP mutant cells. hTERT-CRISPR eliminate or alter many functions of hTERT, but the effects are not equivalent to the loss of RdRP activity of hTERT per se. There is no way the authors can suggest loss of BRCA/FA is synthetic lethal with loss of RdRP specifically.

16. The messages of Fig. 6f-6j are mixed because both ALT+ and telomerase+ cell lines were used. When hTERT is knocked in telomerase+ cell lines, its effects are not specific to loss of RdRP. The interpretation of these panels is quite difficult.

Reviewer #3:
Remarks to the Author:
This manuscript reports on the finding that a phosphorylated form of hTERT reverse transcriptase can function as an RNA dependent RNA polymerase in mammalian cells. RdRP activity was found to be directed to R loops to prevent binding to the DNA strand and help resolve R loops. A synthetic lethal screen identified Fanconi anemia genes as functioning in parallel ion this activity. This finding will have major implications and be of great interest to a large range of scientists interested in genome stability.

p-hTERT was shown to occur in nuclear speckles in several human cell lines including those lacking telomerase activity (ALT cells). Their previous work had shown that phosphorylation of hTERT is necessary for hTERT-mediated RNA dependent RNA polymerase (RdRP) activity but dispensable for reverse transcriptase and terminal transferase activities. They enriched the hTERT and p-hTERT immune complex with antibodies and performed telomerase and RdRP assays – the fractions appear clean as judged by their analysis. However, this is such an important conclusion that it would be good if the authors could show RdRP activity with a recombinant complex to ensure that it is p-hTERT activity and not a contaminating one.

p-hTERT contains a predicted IDR so a procedure was developed to isolate nuclear speckles. Fig 3 shows that the nuclear speckle fraction is the pellet after Triton, Mnase and a salt wash. This enriched MALAT1 and phospho SR proteins. Their analysis suggests this fraction is relatively pure. However, it would be good to get additional experimental evidence to give confidence that this is fraction really is enriched for the speckles. Dynamic liquid-liquid speckles are generally not stable enough for such a cellular fractionation.

They found IP'ed hTERT RdRP activity synthesized antisense transcripts to TERRA RNA in vitro, and

incubation of telomeric repeat RNA-DNA hybrids with hTERT immune complex suggested that hTERT dissolves R-loop structures. Used s9.6 immunoprecipitation and dot blot analysis they assayed R loops in different backgrounds. A double knock out of the hTERT and FA pathway gave a striking up-regulation of R loops. The signal was RNAseH sensitive consistent with a change in an RNA-DNA hybrid. An issue with this experiment is that the dot blot assay is prone to background issues and which strand of RNA is involved cannot be elucidated. Can they supplement this assay with a strand specific sequence technique -to have full confidence in the R loop identification.

A knock-out experiment of hTERT in ALT cells produced heterozygous clones with lower hTERT. RNA-seq analysis using these clones identified which transcripts were altered in expression and GO terms showed enrichment of genes involved in cell cycle and DNA replication. This led to a genome wide CRISPR screen using the cell lines with the mutant hTERT, screening for reduced viability dependent on the mutant hTERT. They found Fanconi anemia and DNA repair genes and validated their effects by suppression of those genes individually by siRNAs. Thus, Fanconi anemia pathway and hTERT appear to function in parallel. This is consistent with their finding that when both pathways mutated the cells were more sensitive to DNA damage agents. This part of the analysis was very convincing.

Overall, this is an important study, but additional experiments would give confidence in what could be a controversial set of data.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

MANUSCRIPT FORMAT – please follow the guidelines listed in our Guide to Authors regarding manuscript formats at Nature Cell Biology.

TITLE – should be no more than 100 characters including spaces, without punctuation and avoiding technical terms, abbreviations, and active verbs..

AUTHOR NAMES – should be given in full.

AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the

names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

ABSTRACT AND MAIN TEXT – please follow the guidelines that are specific to the format of your manuscript, as listed in our Guide to Authors (http://www.nature.com/ncb/pdf/ncb_gta.pdf) Briefly, Nature Cell Biology Articles, Resources and Technical Reports have 3500 words, including a 150 word abstract, and the main text is subdivided in Introduction, Results, and Discussion sections. Nature Cell Biology Letters have up to 2500 words, including a 180 word introductory paragraph (abstract), and the text is not subdivided in sections.

ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see https://www.nature.com/licenceforms/nrg/competing-interests.pdf.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

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Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words. The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human

subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

All Nature Cell Biology manuscripts submitted on or after March 21 2016 must include a Data availability statement at the end of the Methods section. For Springer Nature policies on data availability see http://www.nature.com/authors/policies/availability.html; for more information on this particular policy see http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf. The Data availability statement should include:

• Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here http://www.nature.com/authors/policies/availability.html#data.

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• At a minimum, please include a statement confirming that all relevant data are available from the authors, and/or are included with the manuscript (e.g. as source data or supplementary information), listing which data are included (e.g. by figure panels and data types) and mentioning any restrictions on availability.

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DISPLAY ITEMS – main display items are limited to 6-8 main figures and/or main tables for Articles, Resources, Technical Reports; and 5 main figures and/or main tables for Letters. For Supplementary Information see below.

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All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a

statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

We accept files from the following graphics packages in either PC or Macintosh format:

- For line art, graphs, charts and schematics we prefer Adobe Illustrator (.AI), Encapsulated PostScript (.EPS) or Portable Document Format (.PDF). Files should be saved or exported as such directly from the application in which they were made, to allow us to restyle them according to our journal house style.

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- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.

- Some programs can generate Postscript by 'printing to file' (found in the Print dialogue). If using an application not listed above, save the file in PostScript format or email our Art Editor, Allen Beattie for advice (a.beattie@nature.com).

Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the "unprocessed scans" Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial http://www.nature.com/ncb/authors/submit/index.html#suppinfo; http://www.nature.com/ncb/journal/v14/n3/index.html#ed). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos much be accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – To improve the quality of methods and statistics reporting in our papers we have recently revised the reporting checklist we introduced in 2013. We are now asking all life sciences authors to complete two items: an Editorial Policy Checklist (found

here https://www.nature.com/authors/policies/Policy.pdf) that verifies compliance with all required editorial policies and a reporting summary (found here https://www.nature.com/authors/policies/ReportingSummary.pdf) that collects information on experimental design and reagents. These documents are available to referees to aid the evaluation of the manuscript. Please note that these forms are dynamic 'smart pdfs' and must therefore be downloaded and completed in Adobe Reader. We will then flatten them for ease of use by the reviewers. If you would like to reference the guidance text as you complete the template, please access these flattened versions at http://www.nature.com/authors/policies/availability.html.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from n<3. For sample sizes of n<5 please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

--------- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements ---------

Author Rebuttal to Initial comments

Detailed response to the editors and the reviewers

We thank the editors and the reviewers for evaluating the manuscript. While all reviewers expressed positive sentiments and found our work of interest, they made some suggestions to strengthen the manuscript. In response to reviewers' insightful and constructive comments, we have performed substantial experimentation and have revised the manuscript. We believe that we have now addressed all of the comments from the reviewers and agree that these changes have strengthened the manuscript.

Editorial comments:

"Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. We are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further."

We very much appreciate the editorial team of Nature Cell Biology for summarizing the reviewers' comments to strengthen the manuscript and giving us the opportunity to revise the manuscript. We have now addressed all of the points as below.

"A: Perform further experiments to establish the specificity of the p-hTERT antibody (for IF and IP) (reviewer#1 pts 1,6, reviewer#2 pts 11-12) and test both p-Htert antibodies in the same cell lines (reviewer#1 pt2)"

In the revised manuscript, we have further validated the specificity of TpMab-3 using several approaches. Specifically, (1) we have performed knockdown of hTERT by siRNAs specific for hTERT and confirmed that the treatment with hTERT siRNAs diminished the IF signal of the anti-p-hTERT antibody (revised Fig. 1c) and the IB signal of p-hTERT isolated by IP using the p-hTERT antibody (revised Fig. 1i), and (2) we have tested all p-hTERT antibodies (clones TpMab-1, -3, and -35) in the same cell lines such as 293T, HeLa, U2OS, VA-13 and Saos-2 cells (revised Fig. 1a-d, f, and Suppl. Fig. S1a-c). We have incorporated these new data in the revised manuscript. Please also see the detailed response to the reviewers (the reviewer #1 comment F#1, 2, 6 and the reviewer #2 comment #11-12).

"B: Conduct further experiments to test the proposed model (that p-hTERT specifically associated with TERRA to carry out RdRP function and suppress R-loops), as per reviewers recommendations (reviewer#1 pt 2, reviewer#2 pt 13-16, reviewer#3 paragraph 2, 4)"

As suggested by the editorial team and the reviewers, we have further provided new data on the model of R-loop regulation by RdRP. Reviewer #1 and Reviewer #2 requested that we further confirm that the R-loop regulation with TERRA RNAs occurs in nuclear speckles, since TERRA RNAs are known to be enriched at telomeres (the reviewer #1 comment E#2 and the reviewer #2 comment #13). To address this point, we have examined the localization of TERRA RNAs by FISH and have verified the localization of

TERRA RNAs in nuclear speckles (revised Fig. 4c). The reviewer #2 also stated that it was unclear whether the growth inhibition by genome editing of *hTERT* is related to R-loops (the reviewer #2 comment #14). To clarify this point, we have quantified R-loops with TERRA RNAs in the hTERT CRISPR clones and confirmed that the hTERT-CRISPR clones have higher levels of R-loops with TERRA RNAs (revised Fig. 5d). These results suggested that accumulation of R-loops caused the growth inhibition of hTERT-CRISPR clones.

In addition to this point, the reviewer #2 pointed out that other functions of hTERT might be involved in the synthetic lethality between hTERT and FANC/BRCA genes (the reviewer #2 comment #15). To exclude the involvement of other functions of hTERT, we have overexpressed T249A mutant hTERT, which has single amino acid mutation in the phosphorylation site of hTERT and thus lacks RdRP activity (Yasukawa *et al*., Nat Commun, 2020). When we induced the synthetic lethality in hTERT-CRISPR clones by knockdown of FANC/BRCA genes, growth inhibition of the hTERT-CRISPR clones was rescued by overexpression of wild-type hTERT, but not T249A mutant. Since the alanine substitution of T249A mutant affects only hTERT RdRP activity (Yasukawa *et al*., Nat Commun, 2020), these data strongly suggest that the loss of RdRP activity, but not other functions of hTERT, causes the synthetic lethal phenotype. We have incorporated the data in the revised manuscript Fig. 6g and discussed the point in Results section (revised manuscript page 15).

Reviewer #2 also pointed out that the effect of hTERT knockdown is not specific to RdRP activity (the reviewer #2 comment #16). In our original submission, we inhibited the RdRP activity with a CDK1 inhibitor in FANCF-deficient cells and confirmed that FANCF-deficient cells are more sensitive to the inhibition of RdRP activity (original Fig. 6j). The CDK1 inhibitor has an inhibitory effect on the RdRP activity of hTERT, although hTERT knockdown might affect other functions of hTERT. Therefore, these results imply that the loss of RdRP, but not other functions, is synthetic lethal with FANC/BRCA mutation (original Fig. 6j). To further confirm that the cell death caused by inhibiting hTERT does not depend on inhibition of telomerase activity in telomerase-positive cells, we treated FANCF mutant cells with a telomerase inhibitor. The inhibition of telomerase activity had a comparable inhibitory effect on both FANCF wild-type and mutant cells, suggesting that the cell death through the synthetic lethality in these telomerase-positive cells is not due to inhibition of telomerase activity (revised Fig. 6l). We believe that these data emphasized the importance of RdRP activity in the synthetic lethal pathway

Reviewer #3 requested that we test the RdRP activity with a recombinant protein (the reviewer #3 comment #1 in paragraph 2). Since we agree that these experiments would significantly strengthen the manuscript, we have performed experiments using purified recombinant hTERT (rhTERT) produced by baculovirus expression systems and confirmed that rhTERT proteins exhibited RdRP activity (revised Suppl. Fig. S2d, S3c and S3d). Furthermore, this reviewer #3 suggested to assess the R-loop structures by not only dot blot analysis but also other experiments with “a strand-specific technique” (the reviewer #3 comment #3 in paragraph 4). Accordingly, we have performed DNA:RNA immunoprecipitation (DRIP) analysis and have found that R-loop structures with TERRA RNAs accumulate in the cells with double-knockdown of hTERT and FANC/BRCA genes (revised Fig. 7b). These results from the dot blot analysis and the DRIP analysis were consistent (original Fig. 7a and revised Fig. 7b). Therefore, we believe that

these data further support the mechanism by which accumulation of R-loop structures leads to the cell death.

We agree that the alterations have strengthened the revised manuscript.

"C: Test whether hTERT and p-TERT colocalise in cells (Referee#2 pt 3,9,10 Reviewer#1 Ept2,)"

The reviewers requested to confirm the localization of phosphorylated hTERT (p-hTERT) in nuclear speckles and they specifically suggested that we should test whether pan-hTERT, which includes both unphosphorylated and phosphorylated hTERT, and p-TERT colocalize in cells. Since the antibody specific for pan-hTERT (2E4-2) and the anti-p-hTERT (TpMab-3) antibody are both established from the same species (mouse), it is technically impossible to discriminately perform co-IF staining of pan-hTERT and p-hTERT. Therefore, we have performed the IF analysis using each antibody and compared the signals. We have found that while pan-hTERT exists throughout the nucleus, p-hTERT only localizes in the nuclear structures (revised Suppl. Fig. S4a). These results imply that pan-hTERT and p-hTERT co-localize in the nucleus in some part. We have included the new data (revised Suppl. Fig. S4a) and clarified the point (revised manuscript pages 8-9). Please also see the detailed response to the reviewer #2 comment #3.

"D: All other referee concerns pertaining to strengthening existing data, providing controls(Reviewer#1 pt1, reviewer#2 pt6,8), methodological details, clarifications and textual changes, should also be addressed."

As suggested by the editors and the reviewers, we have provided additional experimental controls in this revised manuscript. Specifically, (1) we have stringently validated the specificity of the p-hTERT antibody for IF and IP analyses in various cells (the reviewer #1 comment F#1, the reviewer #2 comment #1, 2, 6, 11, and 12), (2) we have determined hTERT protein levels immunoprecipitated by the anti-hTERT antibodies in the analyses for examining hTERT enzymatic activities (the reviewer #2 comment #8), and (3) we have consolidated the data about R-loop regulation through the RdRP activity by a method of IP using the phospho-specific anti-p-hTERT antibody (TpMab-3) as well as anti-hTERT antibody (10E9-2) (the reviewer #2 comment #11 and 12).

As described above (editor's comment B), we have incorporated significant amount of additional data to strengthen the model for the control of R-loop structures through the RdRP activity. For example, we have provided additional data on the RdRP activity of hTERT using a recombinant hTERT protein (the reviewer #1 comment F#2, #4, and the reviewer #3 comment #1). We have further demonstrated that TERRA RNAs localizes in nuclear speckles (the reviewer #1 comment E#2 and the

reviewer #2 comment #13). We have further investigated why ALT cells have no telomerase activity and found that hTERT did not associates with hTERC in ALT cells (the reviewer #2 comment #4). We believe that these experiments have substantially strengthened the manuscript.

In addition to these experimental data, we have revised manuscript in response to all reviewers' comments on "methodological details, clarifications and textual changes". We have addressed each point in response to the reviewers.

"E: Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all repeats should be provided."

We have prepared a Supplementary Figure including unprocessed images of all gels/blots and a Supplementary Table including all numerical source data, as suggested.

Reviewer #1:

"A. Summary of results

• ALT (and telomerase-positive [TEL]) cells contain a protein recognised by an anti-phTERT antibody; the quantity is increased in mitosis and reduced by treatment of cells with a CDK1 inhibitor (CDK1i)

• Primers specific for hTERT exons 15-16 detected a band in three ALT cell lines at a level approximately one-third of that in HeLa cells (TEL)

• Protein pulled down by p-hTERT antibody did not contain hTERC RNA, had no telomerase activity (TA), and exhibited RdRP activity; this was decreased by prior treatment with siTERT and CDK1i

• p-hTERT localizes in nuclear speckles (NS); this was disrupted in cells by 1,6-HD, a disruptor of hydrophobic interactions, and by cell lysis in high salt conditions

• NS-enriched cell fractions were found to be depleted of hTERC and to contain protein that was pulled down by anti-p-hTERT antibody, detected by hTERT immunoblotting, and exhibited RdRP activity

• RIP-Seq analysis showed that hTERT associated with telomere repeat-containing noncoding RNAs (TERRA) and that siTERT-treated cells had higher levels of TERRA

• hTERT immune complex (IC) had RdRP activity with TERRA RNA (this appeared to be preferential over RNAs without TERRA sequences); bound to ds-RNAs (detected by J2 antibody); and degraded telomeric repeat RNA:DNA hybrids

• The authors therefore concluded that the RdRP activity in hTERT IC disrupts telomeric R-loops by synthesizing dsTERRA

• CRISPR-induced disruption of hTERT expression in an ALT cell line (U2-OS) decreased proliferation in vitro and tumor growth in immunosuppressed animals and resulted in increased vulnerability to DNA damage (ionizing radiation or DNA damage)

• A genome-wide CRISPR-knockout screen showed that the hTERT-CRISPR cells were dependent on BRCA1, BRCA2 and other FANC genes, and double knockdown of hTERT and FANC/BRCA genes showed increased accumulation of R-loops detected by S9.6 antibody; the hTERT and FANC/BRCA pathways did not appear to be epistatic"

"B. Originality and significance

It appears that this is essentially the only lab that has published on the subject of hTERT-associated RdRP activity since the senior author published the first paper during his post-doc in the Hahn laboratory. This manuscript contains many novel findings, and is a conceptual advance on the previous papers on this subject. If confirmed, this is an important finding about a noncanonical role of hTERT or a protein with which it co-immunoprecipitates.

C. Data and methodology

The quality of the data appears to be high, and the data are well-presented"

"D. Statistics

N/A"

We agree with the reviewer's summary and very much appreciate that this reviewer found the manuscript interesting. We addressed all of Reviewer #1's comments below.

"E. Conclusions

1.The authors mostly assume that hTERT possesses RdRP activity without discussing the possibility that it may be a protein that associates with hTERT."

While we agree that we did not discuss "the possibility that it may be a protein that associates with hTERT", we have previously performed a series of experiments that indicate that hTERT itself possesses RdRP activity. In our previous publication, we reported that phosphorylation of hTERT 249T is essential for the RdRP activity (Yasukawa *et al*., Nat Commun, 2020) and we generated phospho-inhibitory or -mimetic hTERT mutants with the substitution of the threonine 249 of *hTERT* with alanine (T249A) or glutamic acid (T249E), respectively. We confirmed that, while hTERT T249A lacks RdRP activity, hTERT T249E had higher level of RdRP activity. Since only a single amino acid change significantly alters the RdRP activity, these results strongly suggest that hTERT possesses the RdRP activity, even if other factors bind to hTERT. We believe that it is highly likely that hTERT itself but not other factors associated with hTERT directly involves the RdRP activity.

In addition to the published data, however, to further strengthen this point and exclude the possibility of the involvement of protein associated with hTERT pointed out by the reviewer #1, we have now examined the RdRP activity using a purified recombinant hTERT (rhTERT) protein by baculovirus expression systems and confirmed that rhTERT proteins exhibited RdRP activity (revised Suppl. Fig. S2d). Since the recombinant protein purified from a non-mammalian system does not associate with other mammalian factors, it is possible to directly analyze the function of the target protein (hTERT in the case of this study) by using the purified recombinant proteins (IM Rosenberg. Protein Analysis and Purification. 1996). Given that rhTERT had the strong RdRP activity, we believe that hTERT itself, but not other mammalian proteins associated with hTERT, has the RdRP activity.

We have now included the data (revised Suppl. Fig. S2d) and discussed the points in the revised manuscript (page 8). We thank the reviewer for this constructive suggestion and we agree that the alterations significantly strengthen the manuscript. Please also see the response to the comment F#4 of this reviewer #1 on "Suggested improvements" and the comment #1 of the reviewer #3.

"2. I think there is a major problem with the conclusion that the RdRP activity is responsible for R-loop unwinding at the telomere given that it appears to located in nuclear speckles. The authors should clarify this."

In our original submission, we found that hTERT purified from nuclear speckle fractions binds to TERRA RNAs and dissolves R-loop structures with TERRA RNAs (original and revised manuscript Fig. 4). These results suggest that the RdRP activity also functions outside telomeres. However, to further strengthen the data supporting the location of R-loop control, we have demonstrated co-staining of the FISH for TERRA RNAs and nuclear speckles marked by an anti-SRRM2 antibody. We confirmed that TERRA RNAs localized in nuclear speckles (revised Fig. 4d). These results further support our model in which RdRP regulates R-loop structures in nuclear speckles but not telomeres. We thank to the reviewer for pointing this out. Please also see the response to the comment 13 of the reviewer #2.

"F. Suggested improvements

1. Regarding the pTERT IF results (e.g., Figures 1a, b, c, e), visualizing endogenous levels of TERT has been notoriously difficult. It would be desirable to provide a negative control, e.g., a cell in which TERT has been knocked out. Given the difficulties in achieving this with CRISPR, maybe the U2-OS CRISPR clones could be treated with TERT siRNA to produce a negative control."

As the reviewer pointed out, the field of telomere and telomerase recognizes that it is notoriously difficult to detect endogenous levels of hTERT. Therefore, we have carefully validated the specificity of a series of anti-hTERT antibodies (Maida *et al*., MCB, 2014, Maida *et al*., MCB, 2016, Yasukawa *et al*., Nat Commn, 2020, and Matsuda *et al*., J Pathol, 2022). In addition to the previous publications, we further validated the specificity of the anti-p-hTERT antibody (TpMab-3) in our original submission of this manuscript (original Fig. 1d and 1i-k). However, we agree that we did not provide hTERT knockdown or knockout controls, as pointed out by the reviewer #1. We have now performed hTERT knockdown by treatment with siRNAs specific for hTERT. When we treated the cells with siRNAs specific for hTERT twice to suppress hTERT expression efficiently, the treatment with hTERT siRNAs significantly diminished the IF signal detected by anti-p-hTERT antibodies. We incorporated these new data (revised Fig. 1c and Suppl. Fig. S1b).

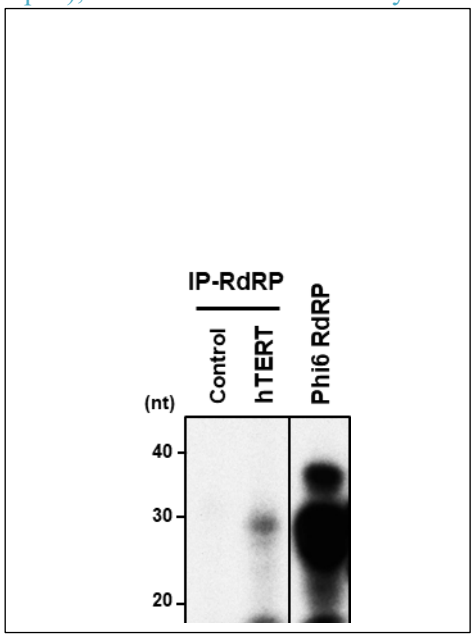
"2. Could the authors comment on the possibility that alternative splicing is involved. The dominant TERT band and the dominant pTERT bands on WB in figures 1f and g appear to be the same size. Is it possible that these are a catalytically inactive spliced form of the protein? The paper that first described

TERT alternative splicing showed that an ALT cell line expressed alternatively spliced TERT (Kilian A, et al. Hum Mol Genet. 1997;6(12):2011-9).”

Researchers have identified various alternative splicing (AS) variants of hTERT mRNA, such as α-, β-, γ-, Δ2, and Δ4-13 variants (Kilian *et al*., Hum Mol Genet, 1997, Yi *et al*., Neoplasia, 2000 and Nucleic Acids Res, 2001, Hrdlickova *et al*. MCB, 2012, etc.). Since ALT cells also express these splicing variants (Kilian *et al*., Hum Mol Genet, 1997 and Hrdlickova *et al*., MCB, 2012), this reviewer asked whether hTERT proteins detected in this manuscript come from the hTERT AS variants. We believe that hTERT proteins detected with anti-hTERT antibodies in the manuscript are not these spliced form of hTERT proteins, because we detected the approximately 127 kDa protein, which is identical to the size of the full-length hTERT protein. Among the AS variants, β-, γ-, and Δ4-13 variants have large deletions (revised Suppl. Fig. S3a), and the deletion in spicing variants such as β-, Δ2 variants causes frameshifting and the subsequent emergence of premature termination codon and nonsense-mediated decay of mRNAs (revised Suppl. Fig. S3a). Thus, these variants would not be hTERT proteins isolated in the IP-IB and IP-RdRP assay. However, there remains a possibility that the α- variant is the approximately 127 kDa hTERT protein detected here, because it has only the deletion of small regions (36 nt) and do not show frameshifting. We therefore determined the α- variant mRNA level with specific primers and investigated its RdRP activity. Although ALT cells expressed the α- variant mRNA as well as the full-length hTERT and β-, γ-, and Δ4-13 variant mRNAs (revised Suppl. Fig. S3b), we observed that the AS variants exhibited no RdRP activity (revised Suppl. Fig. S3c and d). Since we detected high RdRP activity in ALT cells (original Fig. 2d), these results strongly suggest that hTERT detected in this manuscript is the full-length hTERT but not AS variants such as the α- variant. We have incorporated these new data (revised Suppl. Fig. S3) and revised the manuscript (page 8). We thank the reviewer for this important suggestion.

“3. Figure 2 – Has the RdRP assay been validated in any other lab? I note that the 2009 Nature paper has been quite extensively cited (>400 times) but I was unable to easily find a paper that reproduced the assay. Could the authors please comment on this?”

We very much appreciate the opportunity to explain this important issue. We agree that the RdRP assay is a very laborious assay, and the experimental protocol must be followed precisely. Several laboratories, for example Hahn lab (Dana Farber Cancer Institute), Nishikura lab (U Penn.) and Ichimura lab (NCC, Japan), have visited our laboratory to learn the assay, and all of them were able to reproduce the assay. At the same time, they all agreed that the assay is difficult to perform. We speculate that one of the reasons that it is difficult to detect RdRP activity of hTERT is that the hTERT RdRP activity is 100-1000 times less active than that of the Phi6 bacteriophage (Fig. 1, unpublished data). Moreover, in our original publication in the 2009 Nature paper, we utilized recombinant hTERT protein from E. coli (Maida *et al*., Nature, 2009). Since the original RdRP assay requires another difficult process to purify recombinant hTERT protein, many researchers have hesitated to purify recombinant hTERT protein. Other investigators used rabbit reticulocyte systems instead, which we do not recommend. Some laboratories suggested that they could NOT repeat the assay but they did NOT follow our published protocol.



To overcome the difficulties, we established *in vitro* IP-RdRP assay, in which hTERT immune complexes are isolated from cells, followed by RdRP reaction (Maida *et al*., MCB, 2014). This IP-RdRP assay allowed us to detect and determine the RdRP activity of endogenous hTERT. However, the IP-RdRP assay requires specific hTERT antibodies for IP, and the experimental procedures still involve laborious processes that have to be performed carefully. We published the detailed protocol of IP-RdRP assay including videos of each step so that others can reproduce it (Maida *et al*., J Vis Exp, 2018). Furthermore, we believe that the results described in this current manuscript provide insight into why it is technically difficult to detect RdRP activity. In our original submission of the manuscript (original Fig. 3), we found that endogenous p-hTERT localizes in nuclear speckles. In these analyses, we observed that while such speckles are difficult to solubilize under normal conditions, inhibition of the phase separation by 1,6-hexanediol (HD) enables us to easily solubilize p-hTERT (Suppl. Fig. S4i) and the treatment dramatically enriched RdRP activities (Suppl. Fig. S4j). We strongly believe that these findings have made it easier to biochemically analyze the RdRP activity of endogenous p-hTERT, and thus the publication of this manuscript is meaningful and will allow others to perform the RdRP assay.

In summary, we strongly believe that RdRP assay is quite reproducible, if the experimental process is performed correctly and carefully. We continue to be committed to provide help with the assay or teach others if others have difficulty reproducing the assay.

"4. Given that hTERC is not involved, if hTERT is responsible for RdRP activity there is presumably no requirement for holoenzyme assembly so it may therefore be possible to produce highly-purified TERT protein for this assay in a non-mammalian expression system. This would also facilitate the testing of various splice forms and separation-of-function TERT mutants."

The reviewer is correct, and we agree that it is possible to detect the RdRP activity using recombinant hTERT (rhTERT) protein produced in a non-mammalian expression system to confirm that hTERC is not involved for RdRP activity. When we first discovered the RdRP activity of hTERT, we verified the RdRP activity with the rhTERT protein purified from E. coli (Maida *et al*., Nature, 2009). In addition, we found that rhTERT purified from a non-mammalian system such as baculovirus system also exhibits the RdRP activity (Maida *et al*., MCB, 2016). In the current manuscript, we have compared RdRP activity of the rhTERT with that of endogenous hTERT immunoprecipitated from cells, and confirmed that rhTERT exhibits comparable level of RdRP activity (revised Suppl. Fig. S2d). In summary, these results indicate that the RdRP activity of hTERT requires neither a protein associated with hTERT nor hTERC.

As suggested by the reviewer, since recombinant proteins prepared by a non-mammalian expression system are useful for analyses of hTERT AS variants, we purified recombinant AS variant proteins by a baculovirus expression system (Suppl. Fig. S3c). We confirmed that while rhTERT proteins exhibited RdRP activity, recombinant AS variant proteins had no RdRP activity (Suppl. Fig. S3d). These results indicate that the full-length hTERT is essential for the RdRP activity. As we have discussed in the comment E#1 from the reviewer #1, the demonstration that purified recombinant protein exhibits RdRP activity strongly supports the conclusion that this RdRP activity is not the result of an associated hTERT protein.

We have included these data (revised Suppl. Fig. S2d, S3c, and S3d) and discussed the point in the revised manuscript (page 8). We believe that the additional data significantly strengthen the manuscript. Please also see the response to the comment E#1 and F#2 of the reviewer #1 and the comment #1 of the reviewer #3.

"5. hTERT-CRISPR clones: please indicate the location of the 16 bp deletion. What are the predicted consequences of the frameshift? Is the resulting hTERT protein full-length?"

As suggested by the reviewer, we have incorporated the information about the deletion mutation in hTERT-CRISPR clones in the revised manuscript. Specifically, we found by Sanger sequencing analysis that hTERT-CRISPR clone #1 has an allele with 16 bp deletion (853 -868 nt in exon 2) in the hTERT locus in addition to wild-type allele (original Suppl. Fig. S7a), and that hTERT-CRISPR clone #2 has two mutant alleles with 16 bp deletion (848-863 nt or 853 -868 nt in exon 2) in the hTERT locus (original Suppl. Fig. S7b). These 16 bp deletion mutation results in frameshifting and causes a premature termination codon (PTC) (1033 nt from the transcription start site). The PTC in the coding region of

hTERT mRNA would be recognized and degraded by nonsense-mediated mRNA decay (NMD). In fact, hTERT mRNA levels both in hTERT-CRISPR clones #1 and #2 were lower than that in wild-type U2OS cells (original Suppl. Fig. S7c). We agree that the explanation made the manuscript more informative, and we appreciate this reviewer for the constructive suggestion. We incorporated the information in the revised manuscript (pages 12-13).

"6. Minor: the telomerase status of the Capan-1 and TOV-21G cells should be stated."

Capan-1 and TOV-21G cells are known to be telomerase-positive cells. We experimentally confirmed that these cells are telomerase-positive in the revised manuscript (revised Suppl. Fig. S9b).

"7. Minor: On p.9, the sentence commencing "Combination of both treatments ..." is redundant."

We thank the reviewer for pointing this out. We fixed the sentence in the revised manuscript.

"G. References

The authors may wish to comment on the previously published data on gene dependency in ALT cells (e.g., Lu and Pickett, Open Biol 12: 220011)"

We appreciate the reviewer's insightful comment. We have cited the references as suggested by this reviewer and have discussed the point as below (revised manuscript page 22).

"While we have performed a genome-wide CRISPR screening using U2OS cells, a previous report analyzed the gene dependency of ALT cells, indicating that ALT cells depend on genes involved in DNA replication and replication stress response (Lu and Pickett, Open Biol, 2022). In particular, this analysis demonstrated that FANCM was the most essential gene in ALT cells, probably because FANCM suppresses telomeric replication stress in ALT cells (Silva *et al*., Nat Commun, 2019). Consistent to this previous finding, the CRISPR screening in this study identified that FANCM was scored as a gene with high dependency in wild-type U2OS cells, and that FANCM was also essential for hTERT CRISPR clones. These observations imply that FANCM is an essential gene in ALT cells, regardless of hTERT status."

"H. Clarity

The paper is very well-written with relatively few typos. Only minor editing is required to make the English more idiomatic."

We have carefully read throughout the manuscript and have fixed typographical errors.

Reviewer #2:

"They proposed that hTERT can function as a suppressor of R-loops through its RdRP activity independently of telomerase, which is important for the survival of ALT+ cells and formation of ALT+ tumors. Although some of the results of this study are interesting, many experiments are not adequately controlled and carefully interpreted. The conclusions of this study are not yet convincing."

While this reviewer finds that the manuscript is interesting, she/he requires additional experiments. We thank the reviewer for these suggestions and have addressed each points below.

"1. In Figure 1a-1c, the specificity of the p-hTERT antibody is still not fully established. Phospho-specific antibodies are known to have a high tendency to cross-react. The authors must prove that the p-hTERT detects hTERT but not other phosphorylated proteins. The CDK1 inhibitor experiment only shows the phospho site is CDK1 dependent, but it does not show the protein is hTERT. The specificity control in Fig. 1i was only done with western but not IF, and only with U2OS. This is a major issue for the paper."

We previously generated the antibody specific for phosphorylated hTERT (p-hTERT) (clone TpMab-3) and comprehensively validated the specificity of TpMab-3 (Matsuda *et al*., J Pathol, 2022). More specifically, we confirmed the specificity of TpMab-3 by (1) inhibition of hTERT phosphorylation by CDK1 inhibitor, (2) knockdown of hTERT proteins by siRNAs specific for hTERT followed by IP-IB, and (3) *in vitro* kinase assay of hTERT proteins by CDK1-cyclin B proteins with λ-phosphatase treatment to remove the phosphate groups (Matsuda *et al*., J Pathol, 2022). In addition to these previously published results, we further validated the specificity of the p-hTERT antibody in the IF analysis by the treatment with a CDK1 inhibitor in this manuscript (original Fig. 1d in the manuscript). While we believe that these analyses clearly demonstrated that the signals detected by TpMab-3 are specific for phosphorylation, we did not provide an adequate control for IF for p-hTERT as pointed out by the reviewer #2. In our revised manuscript, we incorporated these new data (revised Fig. 1c). Please also see the response to the comment A of the editorial team and the comment F#1 of the reviewer #1.

"2. The two p-hTERT antibodies were not always tested in the same cell lines in different panels. This makes it difficult to judge whether the two antibodies are completely consistent. If the two antibodies give different results, reader need to know and be cautions with interpretations."

The reviewer is correct and we have now conducted the IF analyses with all p-hTERT antibodies (clones TpMab-1, -3, and -35) in the same cell lines such as 293T, HeLa, U2OS, VA-13 and Saos-2 cells. We performed control experiments by using cells suppressed the hTERT expression by siRNAs or inhibited

phosphorylation of hTERT as well as by using unmanipulated cells. All of these experiments demonstrated consistent results with the original submission. We have incorporated all of the data (revised Fig. 1a-d and Suppl. Fig. S1a-c). Please also see the response to the comment A from the editorial team.

"3. Do hTERT and p-TERT colocalized in cells? This is a simple experiment that must be done. It is surprising that the authors have not done it for the manuscript. If hTERT and p-hTERT do not colocalize well, reader should know, and it should be explained."

We agree that it is a very important point whether *pan-hTERT and p-hTERT colocalize in cells. (*To clarify the meaning of "hTERT", we designate unphosphorylated and phosphorylated hTERT as pan-hTERT. The target proteins of each anti-hTERT antibody are summarized in Table. 1.) Since the antibody specific for pan-hTERT (2E4-2) and the anti-p-hTERT (TpMab-3) antibody were both generated from the same species (mouse), it is technically impossible to perform co-IF staining of pan-hTERT and p-hTERT. Therefore, we have performed the IF analysis using each antibody and compared the signals. While we detected pan-hTERT throughout the nucleus, p-hTERT localized only in the specific structures in the nucleus (revised Suppl. Fig. S4a). These results suggest that pan-hTERT localizes even in the specific structures of the nucleus such as nuclear speckles (original Fig. 3). We concluded that pan-hTERT and p-hTERT co-localizes in nuclear speckles. We have included these new data (revised Suppl. Fig. S4a) in the revised manuscript (pages 8-9). We agree that these data have clarified the manuscript. Please also see the response to the comment B from the editorial team.

	Anti-hTERT antibody (10E9-2, 2E4-2)	Anti-p-hTERT antibody (TpMab-3)
Target protein	Pan-hTERT (unphosphorylated hTERT and p-hTERT)	p-hTERT

Table. 1. Target proteins of anti-hTERT antibodies.

"4. In Figure 1d, both VA-13 and Saos-2 cells express both hTERT and hTERC. Why are these cell lines negative for telomerase activity? The relative expression of hTERT and hTERC in different cell lines may not be meaningful for predicting telomerase activity. Fig. 1d can be misleading."

As suggested by the reviewer, we detected both hTERT and hTERC expressions in ALT cells such as VA-13 and Saos-2 cells. Since both hTERT and hTERC are minimum essential for telomerase activity, the reviewer asked "why these cell lines are negative for telomerase activity".

In our original submission, we immunoprecipitated hTERT (designated as pan-hTERT) and phosphorylated hTERT(p-hTERT) with each specific antibody and found that p-hTERT does not form a complex with hTERC in telomerase positive cells (HeLa and 293T cells) (original Fig. 2b). In the revised

manuscript, we performed the same experiment using ALT cell lines. Consistent with the results in telomerase-positive cells, we confirmed that p-hTERT does not associate with hTERC in ALT cells (revised Fig. 2c and Suppl. Fig. S2c) and found that p-hTERT has the RdRP activity even in ALT cells (revised Fig. 2d, e). In addition to this point, we noticed that hTERT does not form a complex with hTERC in ALT cells (revised Fig. 2c and Suppl. Fig. S2c), even if ALT cells express both hTERT and hTERC. Alternatively, hTERT binds to TERRA RNAs in ALT cells (original Fig. 4a and b). Previous reports demonstrated that ALT cells express high levels of TERRA RNAs (Azzalin *et al*. Trends Cell Biol, 2015 and Arora *et al*. Nat Commun, 2014) and that TERRA RNAs associate with hTERC and inhibit telomerase activity (Chu *et al*., Cell, 2017). We speculate that TERRA RNAs might inhibit the formation of telomerase complex in ALT cells and that this might be one of the reasons why ALT cells do NOT show telomerase activity. We have included these data (revised Fig. 2c and Suppl. Fig. S2c) and discussed this point (page 19) in the revised manuscript.

"5. In Fig. 1f, it is surprising that hTERT protein was only detected in nocodazole arrested mitotic cells. Many previous studies have detected hTERT in G1 and S phase cells. Whether the 10E9-2 antibody can detect unphosphorylated hTERT is questionable."

As pointed out by this reviewer, while immunoblotting analysis in the original submission detected hTERT proteins in mitotic cells, hTERT was barely detected in cells without nocodazole treatment (original Fig. 1g and 1h). Therefore, the reviewer asked why hTERT is not detected in interphase and she/he questioned the specificity of 10E9-2. As we intensively validated the specificity of the 10E9-2 antibody in the previous publications (Maida *et al*., MCB, 2014, Maida *et al*., MCB, 2016, and Yasukawa *et al*., Nat Commun, 2020) and have discussed the specificity of the 10E9-2 antibody in the detailed response (the editorial comment A and the reviewer #2 comment #6), we strongly believe that the 10E9-2 antibody specifically recognize hTERT. Nevertheless, we agree that we should discuss hTERT expression in interphase and mitosis.

Consistent with our data, several groups have reported that it is difficult to detect endogenous hTERT in interphase (Tong *et al*., Cell Reports, 2015 and Perera *et al*., Sci Adv, 2019) and that hTERT is enriched in mitotic phase (Xi *et al*., Nucleic Acids Res, 2014). Although the mechanism underlying the efficient detection of hTERT in mitosis had been unclear, in the original submission of this manuscript, we revealed that endogenous hTERT localizes in nuclear speckles in interphase (original Fig. 3a) and hTERT is difficult to be solubilized under normal conditions in interphase (original Fig. 1g and Suppl. Fig. S4i). In these analyses, we found that treatment to disassemble the nuclear speckle structure with 1,6-HD, which disrupts hydrophobic interactions and inhibits liquid-liquid phase separation (LLPS), promotes to solubilize hTERT proteins even in interphase (original Suppl. Fig. S4i). We consider that the manipulation of cells into mitosis, where nuclear membrane disappears and nuclear structures disassemble, is equivalent to the treatment with 1,6-HD (original Fig. 1f). These results suggest that the disassembly of nuclear speckle structures would enable us to solubilize and efficiently detect hTERT

proteins in mitotic cells. In summary, we believe that the solubilization of hTERT proteins, but not the cell cycle, is key for the efficient detection of hTERT. We discussed this point in the Discussion section (pages 19-20).

"6. Fig. 2a and 2c are not convincing because we don't know how much hTERT protein was used in each sample. Because the expression levels of hTERT are different in different cell lines, it is hard to compared immunoprecipitated hTERT complexes directly. Again, the specificities of TpMab-3 and 10E2-3 for IP need to be established. One cannot assume these antibodies are specific for intended targets in immunoprecipitation without evidence."

In our original submission, we examined telomerase (original Fig. 2a) and RdRP activities (original Fig. 2d) of hTERT and p-hTERT proteins immunoprecipitaed with each specific antibody and found that ALT cells exhibited RdRP activity but no telomerase activity. (1) To confirm whether the difference in the expression level of hTERT in each cell affects the measured enzymatic activity, the reviewer requested that we examine hTERT protein levels isolated from the cells. In addition, (2) to ensure that each sample was specifically prepared, the reviewer suggested that we confirm the specificity of these antibodies for IP.

(1) While we presented the data on hTERT levels immunoprecipitated with the anti-hTERT antibody in our original submission (original Fig. 1g), we did not utilized the anti-p-hTERT antibody for IP in these analysis. As the reviewer requested, we have now demonstrated hTERT protein levels immunoprecipitated by both anti-hTERT and anti-p-hTERT antibodies as a loading control. hTERT proteins were recovered in all samples at similar levels (revised Suppl. Fig. S2a).

(2) To investigate "the specificities of TpMab-3 and 10E9-2 for IP", in our revised manuscript, we have conducted IP using these antibodies in hTERT knockdown cells and ascertained that these antibodies are specific to hTERT and p-hTERT for IP experiments (revised Fig. 1i).

In these analyses, we are able to further confirm that the difference in enzymatic activities between the cells is not due to expression levels of hTERT proteins. We incorporated these data (revised Fig. 1i and Suppl. Fig. S2a) and appreciate the reviewer for this constructive suggestion.

"7. The interpretation of Fig. 2b is also problematic. The epitope detected by the TpMab-3 antibody may be incompatible with hTERC binding, but it does not necessarily mean that phosphorylated hTERT cannot bind hTERC. The phospho site is not the only determinant in the TpMab-3 epitope. The conclusion needs to be confirmed by other means."

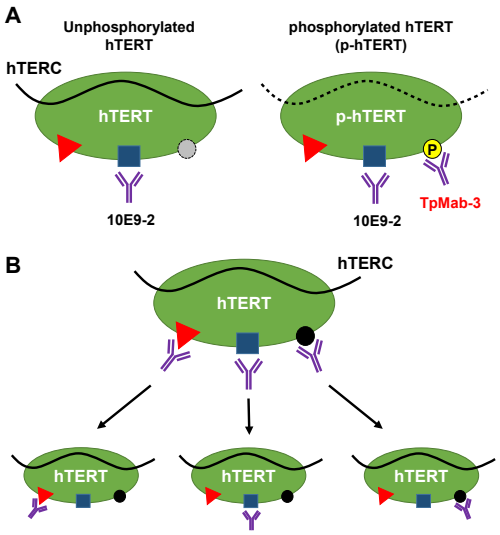


Fig. 2. Target proteins of anti-hTERT antibodies.

A. IP of unphosphorylated hTERT and p-hTERT. p-hTERT immunoprecipitated with TpMab-3 did not associate with hTERC. B. IP experiment isolates the entire target protein and associating RNAs. Each symbol (triangle, square, circle) represents each epitope.

We performed IP of hTERT and p-hTERT proteins with the anti-hTERT (10E9-2) or -p-hTERT (TpMab-3) antibodies and confirmed that p-hTERT immune complex did not contain hTERC (Fig. 2A, original Fig. 2b). The reviewer stated that even if the TpMab-3 epitope did not bind to hTERC, p-hTERT might bind to hTERC using other regions (epitopes). However, we believe that such an IP experiment is supposed to isolate not only the epitope but also the entire hTERT protein associated with other proteins and RNAs (Fig. 2B). In these analyses, p-hTERT immunoprecipitated with TpMab-3 did not associate with hTERC. If p-hTERT associated with hTERC, the TpMab-3 would isolate p-hTERT with hTERC. Therefore, we respectfully disagree with the point ("it does not necessarily mean that phosphorylated hTERT cannot bind hTERC") raised by the reviewer #2. In addition, we confirmed that TpMab-3 specifically recognizes and isolates p-hTERT proteins by immunoprecipitation (revised Fig. 1i, please also see the comment #6 of this reviewer). In summary, we strongly believe that these results support the hypothesis that p-hTERT does not form a complex with hTERC and thus lacks telomerase activity. We would be happy to further discuss the point and revise the manuscript if we misunderstand the point.

"8. The data in Fig. 2d-2f are not meaningful because we don't how much hTERT was used in each sample. Even if RdRP activities were detected in ALT cell lines, it is difficult to judge how robust these activities are. It is impossible to conclude whether RdRP activity is the same or different among ALT+ and telomerase+ cell lines."

The reviewer provided the similar comment as the comment #6 by the reviewer #2 about the original Fig. 2d-2f (Fig. 2e-g in the revised manuscript): she/he asked how much hTERT was immunoprecipitated from each cell for the RdRP assay. In our original submission, we demonstrated hTERT levels used in the original Fig. 2f and g (original Fig. 1i and k). As suggested by the reviewer, we further examined the amount of hTERT proteins in the original Fig. 2e and confirmed that hTERT proteins were recovered at similar levels from telomerase-positive cells and ALT cells (revised Suppl. Fig. S2a). Given that hTERT

in ALT cells (U2OS, VA-13, and Saos-2 cells) exhibited the RdRP activity at the levels comparable to that in telomerase-positive HeLa cells (original Fig. 2d), these results demonstrated that ALT cells show robust RdRP activity at the same level as HeLa cells. We have incorporated these data in the revised manuscript (revised Suppl. Fig. S2a). We thank this reviewer for the meaningful suggestion. Please also see the comment #6 of the reviewer #2.

"9. Given that p-hTERT is localized to nuclear speckles where many phospho-SR proteins are enriched, it is very important to demonstrate the p-hTERT antibody does not cross-react with phospho-SR proteins. This is important for the interpretations of many experiments."

We agree that this is an important point. We have performed experiments to exclude the possibility of cross-reaction between the p-hTERT antibody and phospho-SR proteins. (1) While the treatment with hTERT siRNAs eliminated the p-hTERT signal, it did not affect the signal of phospho-SR proteins (revised Suppl. Fig. S5a). (2) We treated cells with a CLK1 inhibitor KH-CB19 to inhibit phosphorylation of SR proteins, because CLK1 is a specific kinase for SR proteins. The treatment with the CLK1 inhibitor inhibited the phosphorylation of SR proteins, but not that of hTERT (revised Suppl. Fig. S5b). These results indicate that the p-hTERT antibody does not cross-react to phospho-SR proteins. We have added the additional data (revised Suppl. Fig. S5) and revised the manuscript (page 10).

"10. Fig. S3 suggests that p-hTERT undergoes LLPS in both ALT+ and telomerase+ cells. It is inappropriate to conclude that p-hTERT is specifically enriched in nuclear speckles and promotes RdRP activity in nuclear speckles in ALT+ cells (page 9). This is a problem for the model of this paper."

As the reviewer pointed out, p-hTERT localizes in nuclear speckles in both telomerase-positive cells and ALT cells, and the localization of p-hTERT into nuclear speckles is not limited in ALT cells. Indeed, p-hTERT localizes in nuclear speckles in telomerase-positive Huh-7 cells (original Fig. 3a). We agree that the explanation on this point in the original submission might lead to the misunderstanding that p-hTERT exhibits the RdRP activity specifically in ALT cells. To clarify the point, we have revised the manuscript (pages 9 and 10). We thank the reviewer for this suggestion.

"11. In Fig. 4a-4b, the p-hTERT antibody (instead of the hTERT antibody) should be used for IP. If p-hTERT does not associate with APBs/telomeres, it may not bind TERRA as un-phosphorylated hTERT. This is a major issue for the model."

"12. Again, in Fig. 4c-4f, the p-hTERT antibody should be used for IP. All the data DO NOT support the model that p-hTERT specifically associates with TERRA to carry out RdRP function and suppress R-loops. This is a major issue for the model."

We agree the reviewer's suggestion that we should use the p-hTERT antibody for IP in order to verify the R-loop regulation through RdRP activity of p-hTERT. As suggested by this reviewer, we have performed all experiments in Fig. 4a-4f (Fig. 4a-g in the revised manuscript) using the p-hTERT antibody in addition to the hTERT antibody (original Fig. 4, Suppl. Fig. S6d, f, and g). We have now verified that the original results using the hTERT antibody are reproduced when using the p-hTERT antibody (revised Fig. 4). We have re-confirmed the importance of hTERT phosphorylation in R-loop regulation. We have incorporated these data demonstrated by both antibodies into the revised manuscript (revised Fig. 4) and discussed this point in the revised manuscript (page 12). We thank the reviewer for pointing this out.

"13. The model gets very confusing because it is not clear where p-hTERT binds TERRA and suppresses R-loops. If p-hTERT does not localize to APBs/telomeres, does it suppress R-loops in nuclear speckles? Why is TERRA involved in R-loop regulation in nuclear speckles? TERRA is known to be enriched at telomeres in ALT+ and ALT- cells."

While the reviewer stated that "it is not clear where p-hTERT binds TERRA and suppresses R-loops", in our original submission, we biochemically purified nuclear speckle fractions and identified TERRA RNAs in the nuclear speckle-enriched fraction (original Fig. 4a and b). Therefore, we proposed the model in which p-hTERT regulates R-loop structures with TERRA RNAs in nuclear speckles, but not at telomeres. Consistent with our findings, previous reports revealed that not all TERRA RNAs localize at telomeres and some TERRA RNAs exists outside APBs/telomeres, even if TERRA RNAs are enriched at telomeres (Chu *et al*., Cell, 2017 and Arora *et al*., Nat Commun, 2014). However, since we agree that the point raised by this reviewer is important, we further confirmed the localization of TERRA RNAs outside the telomere. More specifically, we have performed a FISH analysis for TERRA RNAs and demonstrated that TERRA RNAs localizes in nuclear speckles (revised Fig. 4c). We believe that these data have strengthened the model of the R-loop regulation in nuclear speckles. We have incorporated the new data (revised Fig. 4c) and discussed this point in the revised manuscript (page 11). We appreciate the reviewer for this important suggestion. Please also see the response to the comment E#2 of the reviewer #1.

"14. hTERT is known to have telomerase independent functions. The interpretation of Fig. 5 is difficult. The CRISPR/Cas9 editing of hTERT was only done in U2OS, and it is not clear whether the effects are specific to ALT+ cells. It is also unclear whether the effects are related to R-loops or telomeres. These experiments do not directly support the model of this paper."

Since previous studies reported that hTERT has telomerase-independent function(s), we agree that we should discuss (1) whether the effect of genome editing against the *hTERT* gene is specific to ALT cells. In addition, the telomerase-independent function of hTERT is not only the RdRP activity, and therefore the reviewer had a question of (2) whether it is related to R-loop regulation through RdRP activity.

(1) We have revised the manuscript to clarify whether the effect of genome editing against the *hTERT* gene is specific to U2OS cells as below. Although we conducted genome editing of *hTERT* in only U2OS cells in the current submission, we genetically engineered the *hTERT* gene in telomerase-positive 293T cells by the CRISPR/Cas9 system to eliminate the RdRP activity in our previous work (Yasukawa *et al*., Nat Commun, 2020). The genome editing of *hTERT* in 293T cells also inhibited tumor formation (Yasukawa *et al*., Nat Commun, 2020). Therefore, we consider that this phenotype of growth inhibition is observed in not only U2OS cells but also other cells including telomerase-positive and ALT cells. As we agree that this is an important point and should be clarified, we discussed the point in our revised manuscript (page 18).

(2) To investigate R-loop structures when editing *hTERT* gene, we have now performed DRIP (DNA:RNA immunoprecipitation) assay and determined TERRA RNA levels associated in R-loop structures. This analysis demonstrates that TERRA RNAs are more abundant in DRIP samples of hTERT CRISPR clones than those of wild-type U2OS cells, suggesting that R-loops with TERRA RNAs accumulate in cells lacking the RdRP activity. We have included these data (revised Fig. 5d) and revised the manuscript (page 13). We thank the reviewer for these constructive suggestions. Please also see the editorial comment B.

"15. The data in Fig. 6 is difficult to interpret because it is inappropriate to use hTERT-CRISPR cells and RdRP mutant cells. hTERT-CRISPR eliminate or alter many functions of hTERT, but the effects are not equivalent to the loss of RdRP activity of hTERT per se. There is no way the authors can suggest loss of BRCA/FA is synthetic lethal with loss of RdRP specifically."

The reviewer pointed out that genome editing of *hTERT* might inhibit function(s) of hTERT other than

RdRP activity. To evaluate the direct involvement of RdRP activity in the synthetic lethal phenotype by inhibition of hTERT and FANC/BRCA genes, we inhibited the RdRP activity, but not other functions, by inhibiting phosphorylation of hTERT (original Fig. 6j). In these experiments, we demonstrated that FANCF mutant cells are more sensitive to inhibition of hTERT phosphorylation (original Fig. 6j), implicating that inhibition of the RdRP activity and the FANC/BRCA pathway are synthetic lethal. To further consolidate the conclusion in the revised manuscript, we have overexpressed wild-type or T249A mutant hTERT in hTERT CRISPR clones. T249A mutant, in which the threonine 249 of hTERT is replaced with alanine, are not phosphorylated and thus lack the RdRP activity (Yasukawa *et al*., Nat Commun, 2020). Although overexpression of wild-type hTERT restored the synthetic lethal phenotype in

hTERT CRISPR clones with FANC/BRCA knockdown, overexpression of the T249A mutant did not rescue the phenotype (revised Fig. 6g). These data suggest the involvement of RdRP activity, but not other function(s), in the synthetic lethal pathway. We have incorporated the data (revised Fig. 6g) and revised the manuscript (page 15). Please also see the editorial comment B.

"16. The messages of Fig. 6f-6j are mixed because both ALT+ and telomerase+ cell lines were used. When hTERT is knocked in telomerase+ cell lines, its effects are not specific to loss of RdRP. The interpretation of these panels is quite difficult."

Since we utilized both telomerase-positive and ALT cells to evaluate the synthetic lethal pathway, the reviewer pointed out that (1) it is unclear whether the synthetic lethal phenotype is specific to inhibition of RdRP activity, and (2) she/he speculated that inhibition of telomerase activity affects the synthetic lethal phenotype in telomerase-positive cells.

(1) As explained in the response to this reviewer #2 comment #15, to confirm the significance and specificity of RdRP activity in the synthetic lethality, we have addressed the point using wild-type or an RdRP-deficient T249A hTERT mutant.

(2) Moreover, to investigate whether inhibition of telomerase activity affect the viability of telomerase-positive cells, we treated these cells with a telomerase inhibitor 6-thio-dG (Mender *et al*., Cancer Discov, 2015). While the treatment of a telomerase inhibitor induced cell death in both wild-type and FANCF mutant cells (revised Fig. 6l), inhibition of the RdRP activity specifically affected the viability of cells with FANCF mutation (original Fig. 6i and k). These observations revealed that inhibition of telomerase activity did NOT induce the synthetic lethality in telomerase-positive cells. We appreciate the reviewer for this suggestion to clarify this point. We have added the data (revised Fig. 6l) and revised the manuscript (page 15).

Reviewer #3:

"This manuscript reports on the finding that a phosphorylated form of hTERT reverse transcriptase can function as an RNA dependent RNA polymerase in mammalian cells. RdRP activity was found to be directed to R loops to prevent binding to the DNA strand and help resolve R loops. A synthetic lethal screen identified Fanconi anemia genes as functioning in parallel ion this activity. This finding will have major implications and be of great interest to a large range of scientists interested in genome stability."

The reviewer stated that "This finding will have major implications and be of great interest to a large range of scientists interested in genome stability." We very much appreciate the positive and supportive evaluation by this reviewer. In addition, the reviewer suggested several insightful and constructive suggestions to strengthen the manuscript. As we understand that the reviewer is very much supportive for the manuscript and encourages us to strengthen the manuscript, we thank the reviewer for these important comments and have addressed each of her/his point as outlined below.

1. *"p-hTERT was shown to occur in nuclear speckles in several human cell lines including those lacking telomerase activity (ALT cells). Their previous work had shown that phosphorylation of hTERT is necessary for hTERT-mediated RNA dependent RNA polymerase (RdRP) activity but dispensable for reverse transcriptase and terminal transferase activities. They enriched the hTERT and p-hTERT immune complex with antibodies and performed telomerase and RdRP assays – the fractions appear clean as judged by their analysis. However, this is such an important conclusion that it would be good if the authors could show RdRP activity with a recombinant complex to ensure that it is p-hTERT activity and not a contaminating one."*

As kindly summarized by this reviewer, we conducted the experiments of IP with anti-hTERT and anti-p-hTERT antibodies and demonstrated that p-hTERT exhibits RdRP activity (original Fig. 2). Since this point is critical for this manuscript, we agree with the reviewer's suggestion that "it is meaningful to ensure that it is p-hTERT activity and not a contaminating one". As for this point, we would remind our published paper, in which we purified a recombinant hTERT (rhTERT) protein by several non-mammalian expression systems such as E. coli and baculovirus (Maida *et al*., Nature, 2009 and Maida *et al*., MCB, 2016). In these previous publications, we found that rhTERT exhibits RdRP activity. To further confirm these results, we have now utilized rhTERT purified with a baculovirus expression system again and compared the RdRP activity of rhTERT with that of hTERT immune complex in the revised manuscript. We observed that rhTERT proteins exhibited high RdRP activity (revised Suppl. Fig. S2d). In general, since usage of a recombinant protein could exclude an effect of contaminating proteins associated with the target protein (IM Rosenberg. Protein Analysis and Purification. 1996), these results indicated that hTERT itself, but not other proteins binding to hTERT, possesses the RdRP activity. We have incorporated the data (revised Suppl. Fig. S2d) and revised the manuscript (page 8). Please also see the response to the comment E#1 of the reviewer #1.

2. *"p-hTERT contains a predicted IDR so a procedure was developed to isolate nuclear speckles. Fig 3 shows that the nuclear speckle fraction is the pellet after Triton, Mnase and a salt wash. This enriched MALAT1 and phospho SR proteins. Their analysis suggests this fraction is relatively pure. However, it would be good to get additional experimental evidence to give confidence that this is fraction really is enriched for the speckles. Dynamic liquid-liquid speckles are generally not stable enough for such a cellular fractionation."*

The reviewer assumed that it is difficult to biochemically fractionate nuclear substructures such as nuclear speckles from cells based on her/his experience and suggested that we should provide additional data to verify the purity of the nuclear speckle fraction. As suggested by the reviewer, we have now determined several precursor mRNAs (pre-mRNAs) and splicing protein levels, which are abundant in nuclear speckles. We observed the enrichment of pre-mRNAs and splicing factors in the nuclear speckle fraction (revised Fig. 3c and d). These findings further support that we were able to purify nuclear speckles. We thank this reviewer for this constructive suggestion.

3. *"They found IP'ed hTERT RdRP activity synthesized antisense transcripts to TERRA RNA in vitro, and incubation of telomeric repeat RNA-DNA hybrids with hTERT immune complex suggested that hTERT dissolves R-loop structures. Used s9.6 immunoprecipitation and dot blot analysis they assayed R loops in different backgrounds. A double knock out of the hTERT and FA pathway gave a striking up-regulation of R loops. The signal was RNAseH sensitive consistent with a change in an RNA-DNA hybrid. An issue with this experiment is that the dot blot assay is prone to background issues and which strand of RNA is involved cannot be elucidated. Can they supplement this assay with a strand specific sequence technique -to have full confidence in the R loop identification."*

In our original submission, we verified the accumulation of R-loop structures in hTERT knockdown cells, detected by the dot blot analysis (original Fig. 7a). As this reviewer stated, we carefully confirmed that the signal was sensitive to treatment with RNase H and that the signal detected by the dot blot analysis are R-loop structures. Nevertheless, since we agree that the dot blot assay may have background signals, we have performed strand specific R-loop analysis by DNA:RNA immunoprecipitation (DRIP) (Sanz *et al*., Nat Protocols, 2019), as suggested by the reviewer. In detail, we first induced double-knockdown of hTERT and FANC/BRCA genes and immunoprecipitated R-loop structures from the cells by DRIP. After treatment with DNase I, the RNA strand of R-loops was subjected to strand-specific reverse transcription (Suppl. Fig. S9h and i). Consistent with the dot blot analysis, this DRIP analysis found that TERRA RNAs associated with R-loop structures accumulated in the cells with double-knockdown of hTERT and FANC/BRCA genes (revised Fig. 7b). We appreciate this reviewer for the constructive suggestion that strengthened our manuscript. We have now included these data (revised Fig. 7b) and discussed the point in the revised manuscript (page 16). Please also see the response to the comment B from the editorial team.

4. *"A knock-out experiment of hTERT in ALT cells produced heterozygous clones with lower hTERT. RNA-seq analysis using these clones identified which transcripts were altered in expression and GO terms showed enrichment of genes involved in cell cycle and DNA replication. This led to a genome wide CRISPR screen using the cell lines with the mutant hTERT, screening for reduced viability dependent on the mutant hTERT. They found Fanconi anemia and DNA repair genes and validated their effects by suppression of those genes individually by siRNAs. Thus, Fanconi anemia pathway and hTERT appear to function in parallel. This is consistent with their finding that when both pathways mutated the cells were more sensitive to DNA damage agents. This part of the analysis was very convincing."*

The reviewer stated that "This part of the analysis was very convincing." We thank the reviewer for understanding our synthetic lethal model and providing these supportive comments.

Decision Letter, first revision:

Our ref: NCB-A51749A

19th February 2024

Dear Dr. Masutomi,

Thank you for submitting your revised manuscript "Maintenance of R-loop structures by phosphorylated hTERT preserves genome integrity" (NCB-A51749A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

Please note that Our articles must have 6 to 8 main figures and they can have up to 10 ED figures. If there are more figures than that, they become supplementary figures. Please convert the "supplementary figures" into "Extended data figures". Please use the full page space to fill each main figure, as each main figure should fill a full page and a font size of no smaller than 6pt Arial or Helvetica throughout for all figures (including main and ED figures).

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Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

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https://orcid.org/0000-0001-9485-1938

Reviewer #1 (Remarks to the Author):

The authors have conducted extensive additional work and have substantially strengthened the manuscript. I do not have additional comments or questions.

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed most of my concerns. The manuscript is now suitable for publication.

Reviewer #3 (Remarks to the Author):

The authors have addressed all my queries satisfactorily

Decision Letter, final checks:

Our ref: NCB-A51749A

1st April 2024

Dear Dr. Masutomi,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Maintenance of R-loop structures by phosphorylated hTERT preserves genome integrity" (NCB-A51749A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Ensuring that each point is addressed will help to ensure that your revised manuscript can be

swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Maintenance of R-loop structures by phosphorylated hTERT preserves genome integrity". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Best regards,

Aimee Frier
Staff
Nature Cell Biology

On behalf of

Sabrya Carim, PhD
(she/her/hers)
Associate Editor, Nature Cell Biology
Nature Portfolio

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Author Rebuttal, first revision:

Point-by-point response to the reviewers' comment

We appreciate the editors and the reviewers for evaluating this manuscript and for their continued interest in our manuscript. We thank you very much for offering to publish the paper (NCB-A51749A).

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Remarks to the Author:

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We thank the reviewer for their recommendation for publication.

Final Decision Letter:

Dear Dr Masutomi,

I am pleased to inform you that your manuscript, "Maintenance of R-loop structures by phosphorylated hTERT preserves genome integrity", has now been accepted for publication in Nature Cell Biology. Congratulations!

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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Please feel free to contact us if you have any questions.

With kind regards,

Sabrya Carim, PhD
(she/her/hers)
Associate Editor, Nature Cell Biology
Nature Portfolio

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