THE EMBO JOURNAL

A single nucleotide incorporation step limits human telomerase repeat addition activity

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 September 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by four referees whose comments are shown below.

As you will see from the reports, the referees all express interest in the findings reported in your manuscript although they also raise as number of issues that you will have to address before they can support publication in The EMBO Journal. In particular, ref #1 encourages the inclusion of data to test the contribution of the pause site mechanism under more physiological conditions (while acknowledging that this will be technically challenging). This referee - together with ref # 3 - also asks for clarifications on the kinetics and the product release assay. Referees #2 and #4 raise few experimental concerns but instead both emphasise the need to extensively elaborate, restructure and rephrase the manuscript to make it more concise and accessible to the non-specialist reader.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

Comments on Ms EMBOJ-2017-97953 (Chen)

The details of the molecular mechanism leading to the RNA templated telomerase mediated telomeric repeat synthesis remains an important subject of investigation, not the least because of potential therapeutic applications down the road. Of course a lot of biochemical groundwork has been laid down on the ciliate RNPs, but the human telomerase harbours specific characteristics that are important for a full understanding.
Recent advances in biochemical analyses and reconstitution assays have allowed important inroads towards the abovementioned goal. One particularly difficult question is how repeat addition processivity (RAP) is achieved, regulated and how this characteristic contributes to telomere maintenance inside cells. Results from the Collins and Stone labs have started to analyze this issue, but it remained unclear why RAP is relatively low for the human enzyme and what determinants contribute to this level of RAP.
The experiments in this manuscript address this issue using extremely clever biochemical setups in vitro. The results allow to conclude that a critical step for RAP resides in the initial template association, or translocation state in which the RNA/DNA bp at position 3 interferes with efficient new incorporation of the next G (position 1) at the 3'-end of the primer at quasi physiological dGTP concentrations. This interference can be overcome by increasing dGTP supply which also causes an increased in RAP. This effect is not dGTP dependent though, but is dependent on specific basepairs surrounding the nt 3 position. Quite surprisingly, the results also establish that the human telomerase can use dGDP as incoming source for G (or dADP for A).
The above conclusions are strongly supported by the high quality biochemistry and the ingenious designs of the assays. As such, this reviewer finds these in vitro experiments very convincing and strong.
Critique:
1) A slight overall shortcoming is the fact that the entire study covers exclusively in vitro data and no in vivo repercussions of the conclusions are validated directly. For instance, would it be possible to mount a system to verify whether G-incorporation from dGDP can occur in vivo? This is a tall order, I do understand, and perhaps not possible. However, applying some genetic tricks in yeast could be used for that?

2) One of the issues not considered in the paper/discussion is the fact that if dGDP can be used as a supply for G, is the intracellular physiological concentration of all the usable G-nucleotides (GDP and GTP) not higher than what is considered here? The results would strongly suggest that GDP-based G-incorporation is significantly lower than the GTP based one. However, since the intracellular GTP/GDP ratio can change quite dramatically depending on stimuli and growth conditions, would that affect the ability of telomerase to mediate telomere extension?

3) Figures 1 and 2: While I agree that the reduction of Km for G1 in the absence of the pause signal is very significant, it would appear that even in the absence, the Km for G1 is 3x higher than that for the G6 and 5x higher than that for the T3. Could there still be some underlying effect that is independent of the pause site signal? Could one measure this by comparing the Kms for the G1 with the G2 (+/- the Pause signal) in assays like that shown in Fig. 1 E?

4) Other issues for the discussion perhaps would be
a) the integration of the ideas in the model of the Wu/Collins 2017 paper. In particular, it seems to this reviewer that the high G1 Km could be lowered by the proposed "active site closing/duplex remodeling" (transition from state 1 to 2 in Fig. 8B) of that paper. This would help the non-specialist reader in the understanding and integration of the conclusions for the big picture.
b) the highly preferred release after position 6 incorporation (see model Fig. 8 bottom) makes the prediction that the 3'-ends on products (or the 3'-ends on chromosome ends) should have a strong

tendency to be in this specific configuration. In a paper by Sfeir/Wright (Mol. Cell 18:1, 131, 2005) this was assessed and only about a quarter to a third of the chromosomal ends had this configuration. The authors could perhaps discuss why this is the case.

Minor Issues:
Page 8, middle: ".. omitting dGTP .." is mentioned twice in the sentence.
Legend Figure S1: "..as mock (A) or with either ...."

Referee #2:

In this manuscript, Yinnan Chen et al. continue JJ-L Chen lab's exploration of the function(s) of the human telomerase "pause site" that is within the template itself. At the heart of the matter are the fascinating and important nuances of the relationship between nucleotide-addition processivity and repeat-addition processivity. In this work, Y. Chen et al. follow up from this lab's earlier paper on the pause signal, Brown et al., PNAS 2014. As with their previous work, the current manuscript is founded upon initial results from using a fragmented-hTR assay that lends itself to studying the template-substrate duplex and nucleotide-addition processivity (but not repeat-addition processivity (RAP) directly since the TF telomerase has lost RAP). The focus of the current manuscript, however, shifts away from template-boundary definition to the role of the "pause signal" on repeat addition processivity, and therefore the authors jump from the conglomerate 3-piece hTR template-free telomerase system to using the standard RRL-reconstituted telomerase with an intact hTR subunit.

It is certainly an ambitious and noble goal to make insights into the molecular mechanism and enzymology of telomerase. It is also useful that the authors herein report Km measurements for telomerase, which is enzymologically rigorous and quantitative. The authors also provide some other new (although rather tangential) results, such as the ability of telomerase to utilize dGDP and dADP. These results are not really surprising for those who study polymerases and reverse transcriptases, as the authors show clearly from their tests of other enzymes.

A central hypothesis for the authors' research presented here appears to be that a template-embedded pause signal is key to repeat addition during telomerase's iterative re-use of its RNA subunit-provided template for reverse transcription by TERT. It is concerning that the present study apparently reverses a main conclusion of the group's previous paper, Brown et al. 2014: in light of additional data provided in this manuscript, the authors now favor "position-specific inhibition" induced by the "pause signal" as opposed to it being "sequence-defined," as stated dozens of times in Brown et al.

Overall, this is very complex, esoteric research on telomerase based on creative approaches. The manuscript starts off in a manner that I found frustratingly confusing, but in later figures things clear up, and I actually really appreciate the model in Figure 8.

Major concerns:

My recommendation for this exciting story is that the authors focus on improving the presentation of the manuscript, by making it clear earlier what the major, over-arching questions are that motivate this research and what will be ultimately advanced by the results of the proposed experiments (i.e., Figure 8). As it is currently written, the first paragraphs of the Results and the first figure start off with an extraordinarily complex experimental, jury-rigged setup: the template-free telomerase with hTR broken into three parts being presented with permutated DNA primers pre-annealed to permutated RNA template fragments, etc. In addition, the rationale provided for undertaking the first experiments in Figure 1 is vexing. For example, should the reader simply accept as fact that "measuring the Km for DNA/RNA hybrid substrates and template-free telomerase" will answer the question of "how the pause signal in the DNA/RNA hybrid affects nucleotide incorporation after template translocation"? It is very hard to evaluate the validity of the logic here (and many other such places), even after seeing the data in Figure 1. Regarding Figure 1, it really must start with a schematic of WILD-TYPE telomerase template sequence, a basic diagram of WT hTR structure, and

a WT telomeric DNA substrate so that the reader can get her/his bearings and clearly see how this ornate TF telomerase system differs from the wild-type situation.

Also, the second sentence of the first paragraph of the Results in particular is vexing without a better diagram or some key forms of additional clarity. Is the idea that sentence 1 of this paragraph refers to the upper diagram in Fig 1A and the second sentence refers to the lower one in Fig 1A? Both cases already have "TAG" synthesized and pause site indicated, etc. It simply isn't sufficiently clear to avoid ambiguity and confusion as to what the authors have in mind as their working model. (It also doesn't help to have two sets of 123456 numbers, missing nt letters in many locations, many colors, ambiguity about what is DNA and what is RNA with many listed letters (Results text uses "d" for deoxy but these are not in figures), etc. Admittedly, it is no easy task to convey such complexity clearly, but unfortunately it simply isn't clear yet for this reviewer and I think any molecular biologist reader should be able to easily evaluate the experiment and underlying rationale for the first figure. Again, this relates to the peril of starting out with such a complex assay condition in the first figure.

Nevertheless, although it is a rough to the Results that begins with details and logic that is hard to follow, at the end of the Discussion it becomes clearer that studying the pause signal is indeed leading to some apparently exciting advances in understanding how the pause site in the template relates to both template definition and first-nucleotide synthesis post-translocation. This is very interesting and parsimonious/compelling to have the same site cause pauses at a distance for two different, but related reasons. For a naïve reader of this manuscript (even a telomerase researcher), seeing Figure 1 makes quite a bit more sense AFTER seeing Figure 8. Thus, it obviously does a disservice to this manuscript to only present the model in Figure 8 in the last paragraph of the Discussion. The authors need to find a way to improve this situation.

The authors should also explain in more detail why it is that a slow step caused by the pause signal ("SLOW" step in Figure 8) is "beneficial" to telomerase (as stated at the end of Discussion). Are such rate-limiting steps inherently beneficial to an enzyme? Can this be demonstrated in some way? Or does the slow step simply provide an opportunity for regulation? Perhaps it helps prevent untoward telomerase activity at broken chromosome ends? Is there evidence for subtle shifts in dGTP concentration in vivo to regulate telomerase?

In Figure 1E, the authors show that simply shifting [dGTP] two- to five-fold higher, from 10 to 20 or 50 μM clearly overcomes the alleged boundary-assistance of the pause site. This modest increase in dGTP concentration overcoming the boundary suggest that the pause site has a weak effect on defining the template boundary. This brings into question the stated rationale for the experiment, which the reader is supposed to accept as fact: "The pause signal embedded in the DNA/RNA hybrid arrests DNA synthesis following two nucleotide additions prior to reaching the end of the RNA template." Thus, my interpretation of the data would be that the supposition on which the experiment was built is questionable, and therefore it is a concerning foothold upon which to make headway.

In summary, this manuscript reports some substantially interesting advances regarding the quantitative details pertaining to the previously reported "pause signal" and it ultimately provides a better, more integrated view now of what this pause site might really be doing. There are also other noteworthy findings herein as well, as mentioned above. As for HOW the pause site does what it appears to do to processivity and boundary definition, that is a critical question that, as the authors point out, remains unsolved and probably will not be determined without biophysical approaches.

Minor concerns:

There are several places where it seems the wrong figure was referred to. For example, the 4th line of page 6.

I was confused by the reference to Fig. 1C on the 9th line from the bottom of page 4.

Middle of page 7, "lanes 6-12" should be "lanes 7-12" no?

Penultimate line of p. 7, it would help to have lane #s.

Referee #3:

Telomerase is an RNA-dependent DNA polymerase with a unique catalytic mechanism that permits the processive synthesis of short telomere DNA repeat sequences during telomere maintenance. In order to achieve repeat addition processivity (RAP), telomerase must complete a telomere repeat, followed by dissociation of the product DNA to promote realignment with the RNA template to prime the next round of repeat synthesis. Chen and colleagues present a detailed set of experiments that dissect the factors that are limiting for RAP in human telomerase. They make use of several of their own recently reported findings, including the presence of a template-embedded pause signal, as well as a template free (TF) telomerase system. Using TF telomerase and a series of RNA-DNA hybrids, the authors measure the apparent Km for dNTP incorporation at each position of the telomere repeat and as a function of the presence or absence of the template embedded pause signal. These experiments reveal that in the TF telomerase system, the affinity for the dGTP to be incorporated at the first position of the nascent telomere repeat is significantly lower than subsequent dNTP positions, and that this low affinity is somehow linked to the presence of the template embedded pause signal. They further show that this feature of the TF telomerase system is not dependent on the identity of the dNTP to be incorporated at the first position, since substitution mutations at this position elicit similar dNTP affinities. The authors next present experiments using a product release assay to demonstrate that RAP is stimulated by increasing levels of dGTP, and that this effect can also be somewhat recapitulated using the hTR51U mutant with elevated dATP levels. This result is consistent with their Km measurements reported with the TF telomerase system. Next, a series of pulse-chase experiments are presented to measure the rates of repeat addition under varying conditions, revealing that elevated dGTP also stimulates repeat addition rates. Again this result holds true for the hTR51U mutant in the presence of elevated dATP. Surprisingly, the authors show that telomerase (and a number of other DNA polymerases) are capable of using dNDPs as substrates during synthesis, a result that appears to distinguish the mechanism of dGDP stimulation of RAP in human telomerase from that previously reported for the Tetrahymena enzyme. Finally, the authors show an interesting set of experiments using a deltaPause mutant, and conclude that in the absence of the template-embedded pause signal the regulatory effects of changing dGTP concentrations is abrogated.

The studies presented in this manuscript are well-designed and reveal interesting insight into the mechanism of RAP in human telomerase. The authors do a commendable job relating results from their highly manipulated TF template system to results with 293T cell reconstituted enzyme. Overall, some of the effects reported in the different experiments appear to be subtle but statistically significant. Finally, the model presented at the end of the study is consistent with the data presented and incorporates findings from other labs in the telomerase field. This study is likely to be of interest to the telomerase community and beyond; however, there are several points that should be addressed prior to publication in EMBO, as described below.

Main points:

1. Regarding the Km measurements reported in Figure 1. No where in the paper could I find a description of experiments that show the reaction being studied is measuring initial velocities, as is required for modeling with MM kinetics. The authors should present data that demonstrates at 60 minutes the rate of product synthesis is still linear with time, or at least state that such a control has been conducted. Also, there should be a more detailed explanation for why they describe a Km apparent, rather than Km. Presumably this is due to the fact that in many of their experiments the Km-app they measure is in fact a convolution of a several step process required to generate the produce being analyzed. This should be made more clear and potential implications of this point on the validity of their Km results and accompanying conclusions should be discussed.
2. In the experiments described in Figure 2, the authors approximate telomerase processivity by measuring the ratio of H/L MW products with a seemingly arbitrary cutoff at ~6 repeats. The authors should describe why this cutoff was chosen in the paper and how the threshold for the H/L ratio impacts their conclusions.
3. Regarding the need to use their 'product release assay' to measure RAP accurately. I am confused

by this experiment. Traditional assays in enzymology that measure processivity typically do so by ensuring the amount of unreacted primer is much greater than the amount of primer that has been extended. In this way, the probability of distributive action of one primer being extended by multiple telomerases is vanishingly small. Yet, the authors take a different approach. Instead, they physically separate the primers that have dissociated by pulling out the telomerase-DNA complexes on beads and then only analyzing RAP for the products in the supernatants. Is the reason this is necessary that the assays are done in a condition where there is not an excess of unreacted primer? It appears they are using 1uM primer for these experiments which is typically sufficient to achieve this condition, given the typically nM concentrations of telomerase generated by reconstitution methods. Is the case somehow different here and if so, the authors should elaborate on this point. More importantly, it would seem to me that the authors method is likely underestimating the true RAP of the enzyme, since they are selectively analyzing only products 3-12 repeats long. In their gels, there is clearly a population of products at the top of the gel under high RAP conditions that is simply not being resolved. Do the authors mean to imply that these products are the result of multiple turnover conditions on the primer? Moreover, isn't the issue that RAP is too fast in these experiments, such that the products are not being well resolved? In this case, perhaps a shorter time point would obviate the need to analyze only the release products in the RAP measurements? This same set of concerns relates to experiments proposed in Figures 3 and Figure 5.
4. In the measurements of repeat addition rate enhancements, there are no indications of the errors of this measurement between replicates? In one case the authors point to the difference between 1.3 and 0.7 repeats/min to support their conclusion (Fig3E), but then claim that a difference between 2.6 and 3.2 repeats/min is 'minor'. These experiments are very clean and I am inclined to agree with the interpretations; however, it is always better to have errors included in the quantification.
5. Regarding the results in Figure 7 on the delta Pause mutants. Can the authors please comment on the changes in overall product profile? In the current version of the manuscript all of the attention in this experiment is focused on the loss of dGTP stimulation without the pause signal, but clearly much has changed about the way the enzyme is behaving with the pause signal mutations.
6. In the working model presented in Fig.8 the authors assign the role of the TEN domain and other processivity factors POT1-TPP1 as inhibiting product release. While this may be true, it is also conceivable that the specialized TEN domain, as well as POT1-TPP1, could influence other aspects the proposed catalytic cycle. Since the current measurements do not address this possibility, the authors may want to revise their model which treats TEN and POT1-TPP1 as only serving some anchor site function.

Minor points:

1. On the top of page 6, the authors refer to (Fig 1C, lanes 1, 4, and 5) but I think they mean Fig2A.
2. On page 5, second paragraph, the authors refer to ' inclusion of 5uM dGTP' but figure shows 10uM dGTP in Fig1E.
3. On page 8, the sentence starting with 'To investigate how dGDP increased....' should be reworded to avoid redundancy within the sentence.
4. Is it known whether Tetrahymena telomerase will incorporate dGDP as well? It would be interesting to see if this difference is really a human telomerase specific phenomenon.
5. Page 10 second paragraph ' 100 folds' should read '100 fold'.
6. Page 10 third paragraph 'DNA/DNA' hybrid should read 'DNA/RNA' hybrid.
7. Same paragraph a few lines down...'a lessen accumulation' should read ' a lesser accumulation'.
8. Page 12 last paragraph...the authors claim that individual nucleotide concentrations below 5uM results in intermediate products accumulation...this statement should be supported by a reference or new data.

Referee #4:

This manuscript describes a series of experiments to investigate the mechanism of telomerase reverse transcriptase (TERT), specifically how telomere repeat processivity is regulated. First, the authors use their previously reported template-free telomerase core constructs (hTERT assembled in rabbit reticulocyte lysate with hTR pseudoknot domain lacking the template, the CR4/5 domain, and various RNA template-DNA hybrids) to determine KM. Second, they report a series of activity assays under different conditions using telomerase purified from human cells that had been

transiently transfected with hTERT and various hTR constructs with wild-type and mutated templates. The authors provide strong evidence that the first nucleotide addition in each repeat is the slow step, thereby limiting processivity, and that this is mediated by a pause signal "embedded in the template" at the first dT-dR base pair of a telomere repeat. In addition, the authors find that telomerase as well as several other investigated DNA polymerases can use NDPs as well as NTPs as substrates. Overall the work is creative and carefully done, and it provides important new insights into the mechanism of telomerase. Following are suggestions to improve the manuscript.

1. The discussion makes little effort to relate the kinetic results to other studies of telomerase mechanism and structure. The authors should discuss whether these results are consistent with, inconsistent with, or do not shed light on models for telomerase catalytic activity such as the hairpin model from Yang and Lee (NSMB 2015) and the SRS (single-stranded DNA retention site) model from Wu, Tam and Collins (EMBO J 2017). In addition to the template embedded pause site human telomerase also contains a physical stop signal which presumably functions in a manner similar to that reported for Tetrahymena telomerase (Jansson et al NSMB 2015; Jiang et al Science 2015). This stop signal would not be functional in the template free telomerase but would be functional in the in vivo assembled telomerase; could this impact the results? [These structural studies should also be referenced, e.g. on page 10, line 12. Wu et al EMBO J 2017 should be referenced on page 12, line 19.]

2. Throughout the manuscript the authors say they "reconstituted native human telomerase in vivo" or "human telomerase was in vivo reconstituted". Since transient transfection of hTR and hTERT was used, the native enzyme may not be what is isolated. This should not affect the results, since for sure hTERT and hTR are present, but the wording should accurately reflect what was done.

3. The hTR template mutants are described in the text using the hTR numbers, but the figures all have the template numbered 123456 in various permutations. This is confusing (although Figure 4 does show those positions if one looks carefully, other ones discussed on page 9 for Figure 7 are not shown).

4. Figure 1A seems to show two pause sites in the template, one at the end of the alignment region and one at the end of the template. Please clarify.
Figure 1B is a little misleading, as the hTR-PK shown only represents a part of the pseudoknot domain used.

5. Figure 4B shows some unusual banding patterns. In lane 5, most of the bands for each repeat are almost equally intense, can the author's explain this? In lanes 7 and 8, why are two dark bands in each repeat almost equally intense? Unusual banding patters are also seen in Figure 5A.

6. It is "well-known" that the chance of misincorporation of a nucleotide is increased if the ratio of nucleotides is far from equivalence. Can the authors tell if nucleotide misincorporation (i.e. wrong nt) could affect any of the results reported here?

7. The use of NDPs by TERT and DNA polymerases is interesting; is it really the case that this has not been previously reported for DNA polymerases?

Minor:
1. Throughout there are grammatical and other wording errors. Please proofread carefully.
e.g. Page 7 "This further supports the nucleotide-dependent stimulation of human telomerase..."
Insert "hypothesis that" between "the" and "nucleotide-dependent" or otherwise clarify this sentence.
Page 4 "We have previously shown that a such transversion mutation...." Such a?
Page 8 "..has previously been reported for a select few some DNA polymerases examined".
Page 9 "seemingly esoteric deoxynucleoside diphosphate usage by human telomerase is quite ubiquitous amongst all the RTs and DNA polymerases..."

2. Please show the WT and delta pause sequences on Figure 7 for clarity.

Point by point response to the reviewers' comments

Referee #1:
1) A slight overall shortcoming is the fact that the entire study covers exclusively in vitro data and no in vivo repercussions of the conclusions are validated directly. For instance, would it be possible to mount a system to verify whether G-incorporation from dGDP can occur in vivo? This is a tall order, I do understand, and perhaps not possible. However, applying some genetic tricks in yeast could be used for that?

We agree with the reviewer that validating our findings in an *in vivo* setting would be interesting. However, such *in vivo* study of enzyme function would be extremely challenging and currently not feasible. For an *in vivo* system, any manipulation of telomerase could have numerous ramifications for the cellular system that would complicate interpretation of the results. Applying the yeast system is also not feasible as yeast telomerase template length, boundary definition and repeat addition processivity are substantially different from human telomerase. In contrast, our *in vitro* system for minimal telomerase reconstitution, together with specific assays that assess individual attributes of the telomerase enzyme or steps of the catalytic cycle, provides unparalleled advantages for investigating the molecular mechanism of human telomerase repeat addition. Our results show that dGDP is a less efficient substrate than dGTP for telomerase and other DNA polymerases. In addition, the dGDP concentration is 5-10-fold lower than dGTP in cells (Bradshaw and Samuels, 2005). Therefore, we expect dGDP usage *in vivo* is less likely. We have revised the manuscript to discuss our view that dGDP is not likely to be utilized as substrate *in vivo*.

2) One of the issues not considered in the paper/discussion is the fact that if dGDP can be used as a supply for G, is the intracellular physiological concentration of all the usable G-nucleotides (GDP and GTP) not higher than what is considered here?

The concentration of dGDP has been reported to be approximately an order of magnitude lower than dGTP (Bradshaw and Samuels, 2005) and thus would minimally change the overall concentration of intracellular deoxynucleotides.

The results would strongly suggest that GDP-based G-incorporation is significantly lower than the GTP based one. However, since the intracellular GTP/GDP ratio can change quite dramatically depending on stimuli and growth conditions, would that affect the ability of telomerase to mediate telomere extension?

We agree with the reviewer that a putative growth condition that promotes extremely high concentrations of dGDP compared to dGTP in the cell could affect telomerase repeat addition activity and telomere length maintenance. However, we are not aware of any such growth conditions and do not have sufficient evidence, nor a feasible system, to address such a possibility.

3) Figures 1 and 2: While I agree that the reduction of Km for G1 in the absence of the pause signal is very significant, it would appear that even in the absence, the Km for G1 is 3x higher than that for the G6 and 5x higher than that for the T3. Could there still be some underlying effect that is independent of the pause site signal?

With the DNA/RNA hybrid substrates that contain the pause signal, we have observed KM values at positions 2 to 6 that range from 4 to 31 μM, all-of-which are significantly lower than the 120 μM value at position 1. In the absence of the pause signal a KM of 22 and 7 μM for nucleotide incorporation at positions 1 and 6 are not unexpected and within the range of KM values found across the template for individual nucleotide incorporations. The likely underlying cause for these variation is the specificity of the telomerase enzyme for the precise telomeric sequence of the DNA/RNA hybrid substrate.

Could one measure this by comparing the Kms for the G1 with the G2 (+/- the Pause signal) in assays like that shown in Fig. 1 E?

Measuring the KM for dG2 incorporation is technically challenging. Our experimental design for KM measurement relies on the incorporation of a radioactive nucleotide for labeling followed by the incorporation of a single nonradioactive nucleotide intended for KM determination. Due to the consecutive incorporations of three dG residues, dG6, dG1 and dG2, measuring the KM exclusively for the last dG2 incorporation would require altering the identity of the nucleotide incorporated by mutations, which would complicate the interpretation of the results.

4) Other issues for the discussion perhaps would be

a) the integration of the ideas in the model of the Wu/Collins 2017 paper. In particular, it seems to this reviewer that the high G1 Km could be lowered by the proposed "active site closing/duplex remodeling" (transition from state 1 to 2 in Fig. 8B) of that paper. This would help the non-specialist reader in the understanding and integration of the conclusions for the big picture.

We have considered presenting a comprehensive working model with all known and proposed functions for the specific steps of telomerase catalytic cycle, which however would be more appropriate for a review article. In addition, presenting a 'review-type' model would distract readers from the central messages of this manuscript. We therefore elect to specifically emphasize our new findings in the working model presented in the new Fig 9.

b) the highly preferred release after position 6 incorporation (see model Fig. 8 bottom) makes the prediction that the 3'-ends on products (or the 3'-ends on chromosome ends) should have a strong tendency to be in this specific configuration. In a paper by Sfeir/Wright (Mol. Cell 18:1, 131, 2005) this was assessed and only about a quarter to a third of the chromosomal ends had this configuration. The authors could perhaps discuss why this is the case.

The reviewer raises an interesting point. Our results predict that telomerase would releases DNA products predominately after the incorporation of dG6 at position 6. The previous analysis of chromosome terminal sequences by Sfeir et al. shows a sharp increase of this specific terminal GGTTAG sequence from only about 25% in telomerase-null cells to about 40% in telomerase-positive cells, which is consistent with our hypothesis that telomerase produces DNA products with this GGTTAG terminal sequence in cells. However, telomere-end processing mechanisms would trim the terminal sequence and produce other five registers of terminal sequences in vivo. We have amended the manuscript to include a brief statement discussing the correlation of telomerase activity with the prevalence of the terminal GGTTAG sequence at chromosome ends.

Minor Issues:
Page 8, middle: ".. omitting dGTP .." is mentioned twice in the sentence.
Legend Figure S1: "..as mock (A) or with either ...."

Minor typographical errors have been corrected and the manuscript was further proofread for errors.

Referee #2:

A central hypothesis for the authors' research presented here appears to be that a template-embedded pause signal is key to repeat addition during telomerase's iterative re-use of its RNA subunit-provided template for reverse transcription by TERT. It is concerning that the present study apparently reverses a main conclusion of the group's previous paper, Brown et al. 2014: in light of additional data provided in this manuscript, the authors now favor "position-specific inhibition" induced by the "pause signal" as opposed to it being "sequence-defined," as stated dozens of times in Brown et al.

Our previous study focused on the template-embedded pause signal, which is "sequence-defined". The present study focuses on the inhibited nucleotide incorporation at the pause site, which is "position-specific" relative to the "sequence-defined" pause signal. We agree that our prior use of the two terms in the text was confusing and may have seemed to present a change-in-view that was unintended. We have revised the manuscript to clarify our use of these terms.

My recommendation for this exciting story is that the authors focus on improving the presentation of the manuscript, by making it clear earlier what the major, over-arching questions are that motivate this research and what will be ultimately advanced by the results of the proposed experiments (i.e., Figure 8). As it is currently written, the first paragraphs of the Results and the first figure start off with an extraordinarily complex experimental, jury-rigged setup: the template-free telomerase with hTR broken into three parts being presented with permutated DNA primers pre-annealed to permutated RNA template fragments, etc. In addition, the rationale provided for undertaking the first experiments in Figure 1 is vexing. For example, should the reader simply accept as fact that "measuring the Km for DNA/RNA hybrid substrates and template-free telomerase" will answer the question of "how the pause signal in the DNA/RNA hybrid affects nucleotide incorporation after template translocation"? It is very hard to evaluate the validity of the logic here (and many other such places), even after seeing the data in Figure 1. Regarding Figure 1, it really must start with a schematic of WILD-TYPE telomerase template sequence, a basic diagram of WT hTR structure, and a WT telomeric DNA substrate so that the reader can get her/his bearings and clearly see how this ornate TF telomerase system differs from the wild-type situation.

We thank the reviewer for the suggestions and have revised the manuscript to better accommodate readers with less intimate knowledge of telomerase function and complexity. Fig 1 and the first paragraph of the Results section have been extensively revised to clearly state the initial question to be addressed in this study. A new Fig EV1 has been added to present a schematic of the wild-type telomerase containing the full-length hTR secondary structure for comparison with the template-free (TF) telomerase system that comprises three hTR fragments, pseudoknot, CR4/5 and the hTR template.

Also, the second sentence of the first paragraph of the Results in particular is vexing without a better diagram or some key forms of additional clarity. Is the idea that sentence 1 of this paragraph refers to the upper diagram in Fig 1A and the second sentence refers to the lower one in Fig 1A? Both cases already have "TAG" synthesized and pause site indicated, etc. It simply isn't sufficiently clear to avoid ambiguity and confusion as to what the authors have in mind as their working model. (It also doesn't help to have two sets of 123456 numbers, missing nt letters in many locations, many colors, ambiguity about what is DNA and what is RNA with many listed letters (Results text uses "d" for deoxy but these are not in figures), etc. Admittedly, it is no easy task to convey such complexity clearly, but unfortunately it simply isn't clear yet for this reviewer and I think any molecular biologist reader should be able to easily evaluate the experiment and underlying rationale for the first figure. Again, this relates to the peril of starting out with such a complex assay condition in the first figure.

We have extensively revised Fig 1 to improve the clarity and consistency of the labels used in the diagrams. The previous Fig 1A has been extensively modified and introduced as the new Fig 2A to reduce the complexity of Fig 1 and clarify the exact experiments performed.

Nevertheless, although it is a rough to the Results that begins with details and logic that is hard to follow, at the end of the Discussion it becomes clearer that studying the pause signal is indeed leading to some apparently exciting advances in understanding how the pause site in the template relates to both template definition and first-nucleotide synthesis post-translocation. This is very interesting and parsimonious/compelling to have the same site cause pauses at a distance for two different, but related reasons. For a naïve reader of this manuscript (even a telomerase researcher), seeing Figure 1 makes quite a bit more sense AFTER seeing Figure 8. Thus, it obviously does a disservice to this manuscript to only present the model in Figure 8 in the last paragraph of the Discussion. The authors need to find a way to improve this situation.

We have simplified Fig 1 and added a new Fig 2A that conveys concepts from the model in old Fig 8 (now Fig 9).

The authors should also explain in more detail why it is that a slow step caused by the pause signal ("SLOW" step in Figure 8) is "beneficial" to telomerase (as stated at the end of Discussion). Are such rate-limiting steps inherently beneficial to an enzyme? Can this be demonstrated in some way? Or does the slow step simply provide an opportunity for regulation? Perhaps it helps prevent untoward telomerase activity at broken chromosome ends? Is there evidence for subtle shifts in dGTP concentration in vivo to regulate telomerase?

The discussion section has been revised to more clearly state the speculated benefit for a telomerase enzyme with limited processive repeat addition is the opportunity to regulate telomere extension in vivo by various DNA-protein interactions: TERT anchor sites and telomerase accessory proteins.

In Figure 1E, the authors show that simply shifting [dGTP] two- to five-fold higher, from 10 to 20 or 50 μM clearly overcomes the alleged boundary-assistance of the pause site. This modest increase in dGTP concentration overcoming the boundary suggest that the pause site has a weak effect on defining the template boundary. This brings into question the stated rationale for the experiment, which the reader is supposed to accept as fact: "The pause signal embedded in the DNA/RNA hybrid arrests DNA synthesis following two [sic, three] nucleotide additions prior to reaching the end of the RNA template." Thus, my interpretation of the data would be that the supposition on which the experiment was built is questionable, and therefore it is a concerning foothold upon which to make headway.

The pause signal plays an important role in defining the template 5' boundary to prevent non-telomeric DNA synthesis beyond the defined template region. Nonetheless, the pause signal alone is not sufficient to completely arrest DNA synthesis at the end of the template and would partly permit non-template sequence used for DNA synthesis in the absence of the physical boundary element, as reported in our previous paper (Brown et al. 2014). The physical template boundary element, comprising P1b and the tethering linker, is another major contributor for defining the 5' boundary (Chen and Greider, 2003). However, we agree with the reviewer that the description of the pause signal in the text was not clear and could lead to confusion. We have revised the wording for the role of the pause signal in defining template boundary to improve clarity.

Minor concerns:

There are several places where it seems the wrong figure was referred to. For example, the 4th line of page 6.
I was confused by the reference to Fig. 1C on the 9th line from the bottom of page 4.
Middle of page 7, "lanes 6-12" should be "lanes 7-12" no?
Penultimate line of p. 7, it would help to have lane #s.

Minor typographical errors have been corrected and the manuscript was further proofread for errors.

Referee #3:

1. Regarding the Km measurements reported in Figure 1. No where in the paper could I find a description of experiments that show the reaction being studied is measuring initial velocities, as is required for modeling with MM kinetics. The authors should present data that demonstrates at 60 minutes the rate of product synthesis is still linear with time, or at least state that such a control has been conducted.

A new Appendix Figure S1 with the results of a time course analysis under low and high nucleotide concentrations has been included to demonstrate the rate of product synthesis remains constant over 80 minutes. Thus, our assays for KM determination at 60 minutes measured the initial velocity of the reaction.

Also, there should be a more detailed explanation for why they describe a Km apparent, rather than Km. Presumably this is due to the fact that in many of their experiments the Km-app they measure is in fact a convolution of a several-step process required to generate the product being analyzed. This should be made more clear and potential implications of this point on the validity of their Km results and accompanying conclusions should be discussed.

"Apparent K_M" was used initially to describe K_M observed with a processive telomerase system, which involves incorporation of three different nucleotides as well as complex reaction steps for processive synthesis of multiple DNA repeats (Lee and Blackburn, 1993). However, in this study, we employed template-free (TF) telomerase that removes processive repeat synthesis and limits the reaction to either two or three nucleotide additions. Therefore, the use of "K_M" would be more appropriate for our kinetic studies with this well-defined non-processive TF telomerase system that catalyzes a straightforward nucleotide incorporation reaction. We have revised the manuscript and figures accordingly.

2. In the experiments described in Figure 2, the authors approximate telomerase processivity by measuring the ratio of H/L MW products with a seemingly arbitrary cutoff at ~6 repeats. The authors should describe why this cutoff was chosen in the paper and how the threshold for the H/L ratio impacts their conclusions.

The ratio of High/Low MW products was to roughly approximate the overall activity of telomerase repeat addition. We have amended the methods section to state that the cutoff was arbitrarily chosen to divide the gel into approximately two even sections. Using different cutoffs give a similar ratio (data not shown). The processivity and rate of repeat addition are specifically measured as demonstrated in Figs 3, 5 and 8.

3. Regarding the need to use their 'product release assay' to measure RAP accurately. I am confused by this experiment. Traditional assays in enzymology that measure processivity typically do so by ensuring the amount of unreacted primer is much greater than the amount of primer that has been extended. In this way, the probability of distributive action of one primer being extended by multiple telomerases is vanishingly small. Yet, the authors take a different approach. Instead, they physically separate the primers that have dissociated by pulling out the telomerase-DNA complexes on beads and then only analyzing RAP for the products in the supernatants. Is the reason this is necessary that the assays are done in a condition where there is not an excess of unreacted primer? It appears they are using 1uM primer for these experiments which is typically sufficient to achieve this condition, given the typically nM concentrations of telomerase generated by reconstitution methods. Is the case somehow different here and if so, the authors should elaborate on this point.

The reviewer is correct that our telomerase primer extension assay was performed under an excess of DNA primers at 1 μM, which would eliminate distributive extension by enzyme turn-overs. However, the issue instead is that, at any given time, there are enzymes processively adding repeats onto a DNA primer. The size distribution of the DNA from these active DNA-enzyme complexes are not identical to the DNA products completely released from telomerase and will influence the measurement of telomerase repeat addition processivity. In fact, the repeat addition processivity quantitated from the enzyme-bound DNA products on the gel is about 50% offset from the values of the released DNA products. By assaying specifically the released DNA products, we assess repeat addition processivity without the 'contamination' of

artificially arrested enzyme-bound DNA products. The results section introducing the product release assay has been amended to clarify the purpose of this assay.

More importantly, it would seem to me that the authors method is likely underestimating the true RAP of the enzyme, since they are selectively analyzing only products 3-12 repeats long. In their gels, there is clearly a population of products at the top of the gel under high RAP conditions that is simply not being resolved. Do the authors mean to imply that these products are the result of multiple turnover conditions on the primer? Moreover, isn't the issue that RAP is too fast in these experiments, such that the products are not being well resolved? In this case, perhaps a shorter time point would obviate the need to analyze only the release products in the RAP measurements? This same set of concerns relates to experiments proposed in Figures 3 and Figure 5.

The assessment of products on the PAGE gel in the 3-12 repeat range was merely to determine the slope (k) for 'products left behind' for the equation: Processivity $= \frac{-\ln 2}{2.303k}$ [20094033]. In our PAGE analysis, the 3-12 repeat products are well resolved to allow quantitation of intensities from major bands. This range is representative for the gel and any portion of the gel could be used where the products were well resolved. We have added this statement to the results section.

4. In the measurements of repeat addition rate enhancements, there are no indications of the errors of this measurement between replicates?

The standard error for the repeat addition rate measurements derived from two independent pulse-chase time-course assays have been included to support the reproducibility of the assay (Figs 3, 5 and 8).

In one case the authors point to the difference between 1.3 and 0.7 repeats/min to support their conclusion (Fig3E), but then claim that a difference between 2.6 and 3.2 repeats/min is 'minor'. These experiments are very clean and I am inclined to agree with the interpretations; however, it is always better to have errors included in the quantification.

With the inclusion of standard error, our updated Fig 3E shows that increasing dGTP from 10 to 100 µM changed the repeat addition rates by nearly 2-fold from 0.86±0.08 to 1.45±0.09 repeat/min. In our new Fig 5E with the hTR mutant 51U, increasing dATP changed the repeat addition rate by almost 3-fold from 1.27±0.03 to 3.15±0.05 repeat/min, while increasing dGTP had no effect on the rate at 2.99±0.01 and 2.95±0.05 repeat/min. The inclusion of an independent replicate consistently supports our conclusion that there was no increased rate for the 51U mutant with increased dGTP.

5. Regarding the results in Figure 7 on the delta Pause mutants. Can the authors please comment on the changes in overall product profile? In the current version of the manuscript all of the attention in this experiment is focused on the loss of dGTP stimulation without the pause signal, but clearly much has changed about the way the enzyme is behaving with the pause signal mutations.

In Fig 7, the telomerase assay of the delta-pause mutant had a distinct product profile, which is presumably due to a shift of the limiting nucleotide incorporation for the mutant enzyme from dG^{1} to the three dA residues. The absence of DNA product released after the dG^{6} incorporation at template boundary indicates that the dG^{1} incorporation is no longer limiting for the delta-pause mutant. Despite this distinct product profile, the delta-pause mutant synthesizes repeats with remarkably higher processivity and rate than the wild-type enzyme, supporting our hypothesis that the pause signal limits the dG^{1} incorporation and the overall repeat addition activity. We have added a short statement in the Discussion to address these factors that potentially impact the product profile for the delta-pause mutant.

6. In the working model presented in Fig.8 the authors assign the role of the TEN domain and other processivity factors POT1-TPP1 as inhibiting product release. While this may be true, it is also conceivable that the specialized TEN domain, as well as POT1-TPP1, could influence other aspects the proposed catalytic cycle. Since the current measurements do not address this possibility, the authors may want to revise their model which treats TEN and POT1-TPP1 as only serving some anchor site function.

The model in our new Fig 9 has been revised to clearly indicate that complete DNA product release is inhibited by DNA-protein interactions and the text further states these interactions are from TERT DNA anchor sites and telomerase accessory proteins.

Minor points:
1. On the top of page 6, the authors refer to (Fig 1C, lanes 1, 4, and 5) but I think they mean Fig2A.

2. On page 5, second paragraph, the authors refer to ' inclusion of 5uM dGTP' but figure shows 10uM dGTP in Fig1E.

3. On page 8, the sentence starting with 'To investigate how dGDP increased....' should be reworded to avoid redundancy within the sentence.

4. Is it known whether Tetrahymena telomerase will incorporate dGDP as well? It would be interesting to see if this difference is really a human telomerase specific phenomenon.

An earlier study (Hardy et al. JBC 276, 4863-71, 2001) from Collins's group showed that dGTP, dGDP or dGMP stimulate Tetrahymena telomerase repeat addition. However, while it is not known whether Tetrahymena telomerase could incorporate dGDP for DNA synthesis, we expect such an ability is conserved in telomerases from different species, as this activity is observed with the many DNA polymerases examined in this study.

5. Page 10 second paragraph ' 100 folds' should read '100 fold'.

6. Page 10 third paragraph 'DNA/DNA' hybrid should read 'DNA/RNA' hybrid.

7. Same paragraph a few lines down...'a lessen accumulation' should read ' a lesser accumulation'.

8. Page 12 last paragraph...the authors claim that individual nucleotide concentrations below 5 uM results in intermediate products accumulation...this statement should be supported by a reference or new data.

Minor typographical errors have been corrected and the manuscript was further proofread for errors.

Referee #4:

1. The discussion makes little effort to relate the kinetic results to other studies of telomerase mechanism and structure. The authors should discuss whether these results are consistent with, inconsistent with, or do not shed light on models for telomerase catalytic activity such as the hairpin model from Yang and Lee (NSMB 2015) and the SRS (single-stranded DNA retention site) model from Wu, Tam and Collins (EMBO J 2017).

Each of these models describe a possible mechanism for the template translocation process, while our study focuses on the first nucleotide incorporation step that follows template translocation and therefor our data cannot provide direct evidence to support or negate either model. We have revised our manuscript to discuss our results in relation to these two models.

In addition to the template embedded pause site, human telomerase also contains a physical stop signal which presumably functions in a manner similar to that reported for Tetrahymena telomerase (Jansson et al NSMB 2015; Jiang et al Science 2015). This stop signal would not be functional in the template free telomerase but would be functional in the in vivo assembled telomerase; could this impact the results? [These structural studies should also be referenced, e.g. on page 10, line 12. Wu et al EMBO J 2017 should be referenced on page 12, line 19.]

The 5' template boundary of TRs from different species are defined by divergent TR structural elements (Chen and Greider, G&D, 2003). We have revised the Introduction section to describe these divergent TR structural elements from vertebrates, ciliates and yeasts with corresponding references cited.

The template-free (TF) telomerase assays in Fig 1 were used to determine nucleotide incorporation kinetics in a simplified system (*i.e.* without the physical template boundary). The K_M values determined using TF telomerase are consistent with the results from assays using the processive wild-type telomerase assembled *in vivo* (*i.e.* with the physical template boundary). The high K_M value for first nucleotide incorporation is a determinant for dGTP-stimulation of telomerase repeat addition processivity and rate.
The sentence on page 10, line 12 was initially referring specifically to the human telomerase RNA with only references related to hTR cited. For a broader reference of TR structures, Wu et al EMBO J 2017 has been cited as the reviewer suggested.

The sentence on page 10, line 12 was initially referring specifically to the human telomerase RNA with only references related to hTR cited. For a broader reference of TR structures, Wu et al EMBO J 2017 has been cited as the reviewer suggested.

2. Throughout the manuscript the authors say they "reconstituted native human telomerase in vivo" or "human telomerase was in vivo reconstituted". Since transient transfection of hTR and hTERT was used, the native enzyme may not be what is isolated. This should not affect the results, since for sure hTERT and hTR are present, but the wording should accurately reflect what was done.

We have revised the statement to clearly state that the wild-type recombinant telomerase was over-expressed in HEK293 human cells by transient transfection for generating hTERT and the full-length hTR containing the internal template.

3. The hTR template mutants are described in the text using the hTR numbers, but the figures all have the template numbered 123456 in various permutations. This is confusing (although Figure 4 does show those positions if one looks carefully, other ones discussed on page 9 for Figure 7 are not shown).

We have modified Figs 4, 5, 7 and 8 that employ template mutations to clearly label these specific mutations by the position in the hTR sequence. Additionally, we have included a new Fig EV1 that denotes the precise location of the hTR template sequence within the hTR structure.

4. Figure 1A seems to show two pause sites in the template, one at the end of the alignment region and one at the end of the template. Please clarify.

We agree that the original Fig 1 was somewhat confusing and overly complicated. To avoid confusion, we have removed the original Fig 1A and added a new Fig 2A in the modified Fig 2 to clearly illustrate the proposed dual functions of the pause signal.

Figure 1B is a little misleading, as the hTR-PK shown only represents a part of the pseudoknot domain used.

The schematic drawing shown in Fig 1B was intended as a cartoon of the pseudoknot fold and not an exact secondary structure of the hTR pseudoknot domain fragment (residue 64–184, as described in materials and methods). To improve clarity, we have added a new Fig EV1A that denotes the overall folding of the full-length hTR and the precise regions employed in TF telomerase.

5. Figure 4B shows some unusual banding patterns. In lane 5, most of the bands for each repeat are almost equally intense, can the authors explain this? In lanes 7 and 8, why are two dark bands in each repeat almost equally intense? Unusual banding patters are also seen in Figure 5A.

The different banding patterns with telomerase template mutants, hTR51U, -50U and -46/52U (Fig 4B), are not unexpected as each mutant incorporates a non-telomeric dA residue at different positions across the template. Under low dATP concentrations, these dA incorporations would become limiting and stall or terminate the DNA synthesis reaction at these different positions. These different stalled/termination positions generate the different banding patterns. The additional band with the template mutant 50U is likely resulted from a lower incorporation efficiency for the dG^6 residue, resulting in the accumulation of DNA products after the dA^5 incorporation under low dGTP concentrations. However, a lower incorporation efficiency for dG^6 and the resulting banding patterns do not impact the conclusion that increasing dATP concentrations stimulate repeat addition for the hTR 51U mutant, and not for the other two mutants. This again indicates that the first nucleotide incorporation is critical for repeat addition processivity and rate.

6. It is "well-known" that the chance of misincorporation of a nucleotide is increased if the ratio of nucleotides is far from equivalence. Can the authors tell if nucleotide misincorporation (i.e. wrong nt) could affect any of the results reported here?

It is true that, under extreme conditions (*e.g.* 1 mM dGTP) and at specific template positions, minor misincorporations of an unintended nucleotide can occur (data not shown). However, our reaction conditions had only 100 μM nucleotide concentrations and we do not observe noticeable misincorporations. For example, in the new Fig 1D (former Fig 1E), an rG residue at the end of the template was not used as template in the absence of dCTP. Moreover, we do not see the expected change in the banding pattern of wild-type telomerase with increased concentrations of a single nucleotide (Figure 2).

7. The use of dNDPs by TERT and DNA polymerases is interesting; is it really the case that this has not been previously reported for DNA polymerases?

The earliest report that we could find regarding DNA polymerase utilizing dNDP as substrate is from a book chapter by Arthur Kornberg (Kornberg, 1957, in *The Chemical Basis of Heredity*). In this book chapter, Kornberg reported that DNA polymerase I is inert with nucleoside diphosphates. This statement seems to have been taken as fact for it was not until almost a half-century later that two articles reported utilization of dNDP by HIV RT and a phage-encoded DNA-dependent DNA polymerase RB69, respectively (Yang et al., 2002, Biochemistry; Garforth et al., 2008, PLoS ONE). Interestingly, both articles reported the inability of the Klenow fragment to utilize dNDP as substrate, which contrasts from our result (Fig 6C, lane 7). We speculate that these discrepancies are due to different dNDP or assay conditions; our assays used higher dGDP concentrations at 100 μM.

Minor:

1. Throughout there are grammatical and other wording errors. Please proofread carefully.
e.g. Page 7 "This further supports the nucleotide-dependent stimulation of human telomerase..."
Insert "hypothesis that" between "the" and "nucleotide-dependent" or otherwise clarify this sentence.

Page 4 "We have previously shown that a such transversion mutation...." Such a?

Page 8 "..has previously been reported for a select few some DNA polymerases examined".

Page 9 "seemingly esoteric deoxynucleoside diphosphate usage by human telomerase is quite ubiquitous amongst all the RTs and DNA polymerases..."

Minor typographical errors have been corrected and the manuscript was further proofread for errors.

2. Please show the WT and delta pause sequences on Figure 7 for clarity.

A diagram with the WT and delta-pause template sequences has been added as Fig 7A.

2nd Editorial Decision 05 January 2018

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees and their comments are shown below. As you will see, they both find that all major criticisms have been sufficiently addressed and recommend the manuscript for publication.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, and congratulations on this nicely executed work!

REFEREE REPORTS

Referee #2:

The authors have made sweeping changes to the wording of the text in many regions, as well as requested changes to Figure 1, which does help improve the manuscript substantially by making the "big picture" clearer. Fundamentally, the conclusions seem generally well-supported by the data. The data quality is high and experiments are well-conceived.

The only question for me is whether this additional quantitative information beyond the authors' 2014 Brown et al. PNAS paper is sufficient for publication in EMBO Journal. It is good that here the focus is the "pause site" rather than the "pause signal" (the latter being the thrust of Brown et al.), and because of the rigorous nature of the experiments being reported here. But clearly the strength of this work is its quantitative nature; it is a paper more about characterization than discovery, but it does this well, and the authors make new biochemical insights that improve our knowledge of important features regarding the fundamentally important and unique telomerase RNP enzyme.

Minor:

"This lower incorporation kinetic" on page 4 - Shouldn't this be "The lower incorporation kinetics"?

Referee #3:

The revised manuscript is significantly improved both in terms of clarity and technical

improvements in response to the reviewer comments. Therefore, I am am satisfied with the revised manuscript, which is suitable for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Julian J.-L. Chen
Journal Submitted to: EMBO J.
Manuscript Number: EMBOJ-2017-97953

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

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	N/A
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Quantification was performed semi-autonomously using Quantity One (Bio-Rad) software with identical areas used to measure product formation intensities across conditions and assays. Recovery controls ensured similar recovery and replicates to ensure repeatability. ImageJ (NIH) software used for determining modal bands for rate determination.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Quantification was performed semi-autonomously using Quantity One (Bio-Rad) software with identical areas used to measure product formation intensities across conditions and assays. Recovery controls ensured similar recovery and replicates to ensure repeatability. ImageJ (NIH) software used for determining modal bands for rate determination.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Statistical tests were not necessary as error was vastly smaller than the differences between values.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	N/A
Is there an estimate of variation within each group of data?	Replicates were performed for all experiments to ensure reproducibility with standard error to the mean (standard deviation divided by the number of replicants) used to ensure repeatability.

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Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	N/A
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A

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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A