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Human Telomerase Specialization for Repeat Synthesis by Unique Handling of Primer-Template Duplex

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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 21 November 2013

Thank you again for submitting your manuscript on telomerase DNA handling for consideration by The EMBO Journal. We have now received the reports of three expert referees, which you will find copied below. As you will see, all three referees consider your findings potentially interesting and important for the field, and acknowledge the technical quality and standards of the presented experiments and approaches. At the same time, however, referees 2 and 3 do raise important concerns regarding the reductionist biochemical approaches and the decisiveness of the biological conclusions that can be drawn from them. In our view, these concerns currently preclude publication in The EMBO Journal as a broad general journal; nevertheless we note that the referees offer a number of constructive criticisms and suggestions that may help to strengthen the relevance of your results for understanding of the native telomerase complex. I understand that extending the study along these lines may not be trivial and will likely require a substantial further amount of time and experimental effort, but I agree with the reviewers that only with such further support for the physiological significance would the study become a highly compelling candidate for The EMBO Journal. Thus, I would like to give you the opportunity to submit a revised manuscript addressing the comments of all three referees, using the link below. Please be reminded however that it is our policy to allow only a single round of major revision, making it essential to carefully respond to all points being raised at this stage, and to convince the referees with additional data and clarifications, in order for the study to ultimately warrant publication. Furthermore, when preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised 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 this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1:

Wu and Collins break new ground regarding the detailed mechanism by which human telomerase engages with its DNA primer and catalyzes repeat synthesis. They first design and validate a new two-piece TERT (the N-terminal TEN domain separated from the RNA-binding/catalytic portion of the protein) and a two-piece TR (template added in trans). This allows them to show that it's the core RNP (excluding the TEN domain) rather than the TEN domain that recognizes the ssDNA primer. They present the first direct primer-footprinting assay that gives insight re. how much of the 5' end of the primer is engaged with the enzyme following active extension. These results lead to a new model for the catalytic cycle of human telomerase.

In general, the experiments are creatively designed, well controlled and beautifully implemented. There are very few labs in the world that could produce such clear data on this system. Although this paper will be most appreciated by those who work on the detailed enzymology of telomerase, I would hope that it would also attract the attention of polymerase enzymologists and structural biologists more generally.

Major suggestions:
1. The Finger and Bryan paper entitled "Multiple DNA-binding sites in Tetrahymena telomerase" (NAR, 2008) seems especially pertinent to the current work, even though it relates to telomerase from a different organism. Those authors already showed that the TERT core more than the TEN domain bound DNA. This work could be cited to good advantage in both the Intro and Disc.

2. Fig. 1E, 2F and 4C, the Relative Activity values are given to 2 sig figs. What are the uncertainties in these numbers? How many independent expts were performed? Should the values of 1.9 and 3.1 (Fig 4C) be reported instead as 2 and 3? It's fine if repeats of the experiment gave the same trend but the values were enough different that they can't be averaged. But the reader needs an indication of reproducibility.

3. Fig. 8B and p. 17 middle paragraph, "The lengths of protected products generally increased with synthesis across the template." Does this imply that the 5'-tail of the primer does not move relative to TERT? That would be my interpretation. The authors should state theirs. What if they allow translocation? Does the protected region then jump back down, or does it continue to increase? This could give major mechanistic insight.

4. I suggest deletion of the P1 experiments (bottom of p. 11 and Fig. 3B,C) unless the authors perform the compensatory base-pair changes for P1 helix. That is, instead of just mutating the bottom strand, one needs to mutate the top strand by itself or mutate both to give compensatory bp changes. In the absence of this information (which is standard way in the RNA field to establish that a structure exists), one has no confidence that the hTR 18-56 RNA is even forming the P1 helix.

5. I suggest deletion of the bottom half of p. 20, the speculations about whether template-primer base-pairing accumulates of stays constant during synthesis. The authors' argument 1 is not totally convincing, because we don't know what else a mismatched primer might be doing that interferes. Their argument 2 is unconvincing, because the accumulating product could be forming a loop.

Minor point: On the Fig. 9 model, it'd be nice to have one final diagram to show reengagement for the next round, or alternatively an arrow from iv back to i.

In the title, the hyphenated word "template-hybrid" is nonstandard. Please consider "template-primer duplex."

Referee #2:

In this manuscript, Wu and Collins extend reductionist study of human telomerase function in vitro, and then leverage the new requirements for the semi-functional system to investigate contributions to single-repeat addition by domains of the RNA and TERT. The most groundbreaking finding is the surprising conclusion that the TEN domain of human TERT contributes some additional activity to template-directed nucleotide addition even on a 6-nt substrate primer (i.e., with no 5' extension that would have been expected for the TEN domain "anchor site" to bind and contribute function). The authors also report, using a templateless pseudoknot-CR4/5-tethered hTR allele, that human telomerase RNA 5'-of-template nucleotides (38-45) in a trans-template context are more important to single-repeat synthesis than nts 3' of the template (57-63). Furthermore, the authors see some template boundary breakdown when the template sequence itself is mutated. Finally, the authors use Exo VII digestion of telomerase-substrate-hybrid complexes to determine that an average of ~18 nts (with a range of ~14-20) are protected by the minimized core RNP.

Overall the quality of the raw data is high and the figures are simple and visually easy to digest. The manuscript is also generally clearly and succinctly written, despite the highly complex experimental conditions, presuppositions about truncated/trans-piece telomerase RNPs' functionality, and the logic and rationale being a bit of a tight rope walk given its intellectual level making it a challenge for the authors to avoid esotericism. There are several novel interesting assays and findings reported related to the pieces of telomerase RNA and use of trans pieces and hybrids (and at the same time also fusing the hTR trans pieces, which expectedly works given the pieces are known to work in trans), the DNA-telomerase exonuclease assay, etc. Furthermore, the unexpected results that the authors obtain with their engineered system to investigate the issue of the TEN domain functioning to apparently directly influence the action of the catalytic core of TERT are important to publish for the telomerase field to consider and address.

This carefully crafted manuscript and the admirably reduced in vitro system the authors have developed allow for some very interesting experiments. Although the "sensitized domain separation-of-function assays" developed to address function of the TEN domain and anchor site are reasonable to make headway on the issue, the degree to which the in vitro data support the conclusions overall is not very strong and the reliance on the amalgamated-fragment telomerase in vitro system is too heavy for EMBO. I elaborate more on the reasons for the concerns below. Overall, claims regarding the TEN domain and TERT anchor site are insufficiently supported without additional experimental approaches and controls to directly address the many alternative explanations that can be invoked to explain the observed surprising results.

Major concerns:

1. Many of the major conclusions rely on a clever, albeit compromised and fragmented telomerase RNP system, as well as quantitatively small (and in at least one case nonexistent) differences in results between experimental conditions. There is frequently no indication of the reproducibility or accuracy of the relative telomerase activity results (e.g., Figures 1E, 2F, 3B, 3D, 4C, 5B). I found it particularly disconcerting that in Figure 4 the telomerase activity in lane 2 (listed as 1.9%) is referred to as "low but detectable" whereas 1.5% and 2.4% values in lanes 7 and 9, respectively, are referred to as "undetectable." Addressing this issue will require more than rewording, since the take-home message from the data seems, at this point, unsupported and subjective. The additional facet of this overarching issue of quantitative claims is that several of the most important findings rely on accuracy of values that approach zero. Subtraction of telomerase activity background signal is a highly inaccurate, subjective process even with the most modern methods and results such as the 17-fold increase one calculates when comparing lanes 1 and 4 of Fig. 6A are highly reliant on the quasi-zero value determined from lane 1. Doing the background subtraction reproducibly the same way could result in the reasonable apparent precision (reasonable error bars) in Figure 6B, despite being

fundamentally and biologically inaccurate. Even with the cleanest gels, the background signal is likely to be many-fold greater than the actual signal in this lane. Thus, as is the case with multiple conclusions in this report, reliance on a single experimental approach and small-scale quantitative differences in results between conditions - particularly in an ornate pieced-together and compromised telomerase assay system - makes it quite difficult to draw solid conclusions.

2. While the authors have developed commendable further-minimized conditions sufficient for few-nt primer extension by human telomerase functional elements, there is complete reliance on the compromised, pieced-together in vitro systems they also are co-reporting here. With the heavily mutated/truncated hTR allele, trans pieces, RNA-DNA hybrids, and unnatural-sequence DNA primers (e.g., poly-T 5' end), the conditions are far from wild type at the outset, and then walk yet further away as the research progresses. The authors do not provide data verifying that the system they propose to have controlled is really behaving as expected. For example, when P1 nts are provided as part of the template fragment, is it known that P1 actually forms? Furthermore, as an indication of how functional or compromised the trans-piece RNPs are, how "active" are they compared to wild type for the nt-addition activity relied upon in this study? Are TEN and CORE portions of TERT fully folded and functional despite being severed from each other? What have the severe truncations of conserved regions of the hTR RNA potentially caused that we might not yet understand? Overall, it is very hard to know if, when an additional variable is examined in this ornate in vitro system and found to cause a change, it reflects a physiologically relevant function. In other words, the work relies on a thick layer of presuppositions and once a new result is built on the existing set, it is then used as the basis for testing the next major variable. Thus, it is hard to interpret the results based on the authors' rationale.

In my opinion, it would greatly improve the work to return to wild-type or quasi-wild-type factors in vitro (and ideally to human cells for EMBO) to test the basic "take-home" message(s) from the in vitro experiments, in order to show that the conclusions are not merely based on artifacts related to the collection of variables introduced by the fragmented system. Thus, although the system set up by the authors may be ornately elegant, it is very hard for the reader to assess biological relevance from the limited extent of data and controls included in the work in support of each conclusion.

3. Another major concern is the non-telomeric poly-T 5' ends of primers used throughout the work, as it is centrally important to TEN domain and anchor site conclusions. If one does not provide the biologically relevant 5' telomeric DNA sequence yet relies on conclusions based on absence of TEN domain and anchor site function using these poly-T 5' ends, it is - at minimum - vexing and, at least for this reviewer, quite disconcerting. How do poly-T 5' ends influence the conclusions being drawn from the experiments about the TEN domain and anchor site involvement in telomerase mechanism, etc. Does human TERT TEN domain bind poly-T ssDNA? How about 1-4 repeats of (TTAGGG)? And does it bind telomerase core RNP without or with DNA added? More experiments, discussion of this issue in the text, and increased referencing of TEN and anchor-site literature seems critical to deal with this confounding issue.

4. Does the purified TEN domain reported by the authors bind DNA by filter binding or gel shift? This should be tested and reported. Does it co-purify with the telomerase RNP when added to the RRL prior to pull-down? If it affects telomerase activity and is not binding 5' extensions of DNA substrate, it should co-purify with the RNP and data supporting this directly should be provided.

5. Also related to the issue of TERT anchor site DNA interaction, published reports such as those of Wyatt et al, 2007 and 2009 from Tara Beattie's group; Lue and Li, 2007; Moriarty et al, MCB 2005 from Chantal Autexier's lab; Hammond papers from 1997 and 1998 from Tom Cech's group; Bairley et al. 2011 from Kathy Friedman's lab, all seem highly relevant, yet none of these papers were cited. Also, the findings linking human telomerase action to 5'-proximal TTAGGG-related sequences in human patients by Morin (Nature, 1991) should be discussed in relation to the unexpected findings in this work about the anchor site and TEN domain.

6. There should be thorough discussion of the data in this work as compared to the data and model reported by Zaug, Podell and Cech, NSMB 2008, including discussion of the relevance of Leucine 14 (as well as testing of L14 mutants), since this residue is apparently conserved in hTERT TEN domain.

7. The logic of the experimental design is not obvious in several instances (and over the course of the 9 figures some experiments seem tangential). Why were nucleotide substitutions within the template attempted when investigating boundary function in Figure 3? Was it expected that the template sequence would have such an effect based on Drosopoulos et al. 2005 and what is the mechanistic explanation/model the authors have for this?

Another example is in Figure 5: why is P1 relevant to tests of TEN domain function on 5' extended primers (and extending them by 21 T residues rather than telomeric TTAGGG sequence)?

8. The title of the manuscript is not particularly informative; could it be more impactful and focused on the most prominent conclusion or two? As it stands, it suggests that the manuscript is spread too thin with multiple claims, often supported by too few experimental results and controls.

Minor concerns that should be addressed:

Why not include wild-type control(s) "across the board" to allow the reader to compare relative activity amongst telomerase RNPs between experiments/figures? Connecting the findings and mini-fragment systems developed here to the cellular condition would help the readership determine the degree to which the impressively and extensively reconfigured telomerases are functional, as well as more precisely what ways they have compromised function.

The dependence of telomerase on RNA 5' vs 3' of the template should be discussed, as there are several reports of tests of this that have been performed on ciliate (where the TRE was identified and "accordion" model proposed) and very recently also yeast telomerase RNA in vitro and in vivo.

Fig 2D: Core TERT + hTRmin in RRL but without TEN is missing. It should be included for comparison.

Figure 4D: The model drawn is highly speculative, and furthermore based on very subtle differences in activity in lanes of 4C, as mentioned above. Although they are helpful, should models be (a necessary) part data figures in order for results to be interpreted?

Citation(s) are needed for the origin and reported dysfunctionality of the hTERT D868A mutation. Also, the text did not state that this Asp mutant should be completely inactive, assuming that is the case and this is why it was tested as a control. (Not all Asp residues in the RT domain disrupt TERT function as shown by Lingner et al., Science 1997, but hTERT D868A appears to correspond to Sc TERT D670A or D671A; mutations which do cause senescence.)

Line 2 of p8; as written, it sounded to me like dGTP and dTTP were BOTH labeled with 32P until I inspected the annotation of the figure.

Hyphenation related to compound adjectives should be addressed in many places throughout manuscript: e.g., while they are not in the manuscript, it is more appropriate to include hyphens in phrases like "trans-template RNA", "RNA-template oligonucleotides" etc.

Additional suggestions:

Fig 1B: Quantify D868A abundance vs. WT and report relative levels

Referee #3:

The study described by Wu and Collins describes the use of a protein and RNA trans complementation system to dissect functional contributions of conserved RNA and protein domains to telomerase function. In addition, the manuscript includes a novel physical footprinting method used to map the 5' boundary of protein-DNA contact within the telomerase-substrate complex. There are two major findings in this manuscript that are well-supported by the data, both of which were

unexpected based upon previous literature. First, the authors provide compelling evidence that a major function of the evolutionarily conserved TEN domain in hTERT is to stabilize short RNA:DNA hybrids in the telomerase active site, in addition to its previously assigned role in mediating interaction with the 5' end of the DNA substrate. Second, using the novel exonuclease protection assay, the authors provide the first direct physical data for the ultimate point of contact between the 5' end of the DNA substrate and the telomerase RNP. Surprisingly, this contact, which has previously been suggested to reside within the TEN domain is in fact within what the authors refer to as the 'core' TERT fragment (corresponding to hTERT TEN).

These two major results are accompanied by a variety of additional experiments utilizing various mutant hTR and DNA primer sequences, and catalytic activity as the functional readout. In the case of each of these experiments the authors infer conclusions that, while consistent with the data, are somewhat less compelling given the extensive manipulation of the native telomerase complex.

Taken together, the authors present a novel model for DNA handling during telomere repeat synthesis by human telomerase. The novelty of the results presented by Wu and Collins merits publication in EMBO Journal; however, there are several issues that should be addressed prior to publication.

Major comments:

1. It is interesting that linking the PK and CR4/5 hTR fragments functions so well to reconstitute telomerase activity. However, there is not a direct comparison between this novel RNA construct, the full-length hTR, and the commonly employed two-piece PK + CR4/5 domain reconstitution. It would be nice to see a direct evaluation of these reconstitution approaches side by side, both in terms of telomerase assembly efficiency and catalytic activity, to better understand how the authors arrived at a linker of 14 nt to be 'optimal'?

2. It is striking that the trans TEN complementation system with bacterially expressed TEN domain and RRL reconstituted telomerase RNP only works when the TEN domain is present during the hTERT translation reaction. The authors provide only one of several possibilities for this important observation; namely, that some component of the complex is mis-folded and the co-folding in the RRL is required to produce activity. What is meant by 'co-folding'? As an alternative, is it possible that there are post-translational modifications of the TEN domain that are occurring in the RRL which may account for this difference? If this were to be the case, it would be interesting to know what these modifications are and how they might relate to telomerase function in vivo. While answering these questions is clearly beyond the scope of the present manuscript, the authors should spend a bit more time discussing the potential implications of the requirement for a very specific order of addition protocol to recover activity in their system.

3. On a related note, this reviewer is curious what is meant by 'an excess of TEN domain' in the methods section describing the TEN trans complementation approach? Are these complexes assayed for telomerase activity directly in the RRL reaction, or are they immunopurified from the lysates? Either way, it would be interesting to know more about the nature of the TEN interaction with the rest of the complex since it is no longer covalently tethered to the rest of the TERT polypeptide? In other words, are the experiments done in the presence of a large excess of soluble TEN domain, or is a single TEN domain stably interacting with the hTERT TEN +minhTR fragments as drawn in the models? A more detailed characterization of the TEN trans-complemented enzyme complex is important information for the reader to better interpret the results of the presented experiments. How would the interpretations of the results change if one were to assume a rapid exchange of the trans TEN domain? These points should be discussed unless there is direct evidence suggesting a stable association of a single TEN domain with the rest of the complex.

4. I am curious whether having the large MBP tag on the TEN domain, which I believe more than doubles its molecular weight, might have an impact on the ability of TEN to complement in trans under certain sensitized conditions? Is there a practical reason why the tag was not removed for these experiments, which would seem to be preferable? And is there reason to believe that some of the trans TEN complementation results would be effected by the presence of the MBP tag?

5. There are several places in the manuscript where the author's choice of which effects are 'substantial' and which are not seems somewhat arbitrary and would benefit from either: (i) a more statistically rigorous treatment of the data, together with error estimates, so that the reader may better appreciate what effects are statistically significant; or (ii) an explicitly more qualitative conclusion in the text. For example:

a. In Fig.4C, the authors describe the telomerase activity observed in the absence of the PK domain to be low but detectable and quantify this activity as 1.9% of the wild type enzyme. Then in the same panel, lane 7, the authors claim that in the absence of trans TEN domain there is no observable activity in the absence of the PK domain, but the relative activity is now 1.5% of wild type. This would seem to suggest that these activities are within the noise of the measurement, and should not be taken as significant in either case. That said, visual inspection of the gels does suggest the author's conclusions are correct, but there may be an issue with the data treatment protocol. Perhaps the authors can use freely available software from the Herschlag lab (SAFA) to integrate the relative amount of each band present, correct for specific activity differences due to sequence content, and then present the data in graphical form? This may lead to more reliable numbers in situations where activity is very low and close to background.

b. A second example is in Fig. 8A, where the authors present ExoVII cleavage data for the full-length TERT vs. the Core and demonstrate that the TEN domain is not required for protection of ~18nt of ssDNA in the complex. The statement is then made that the products appear 'more evenly spaced' with the Core enzyme compared to the Full-length TERT. Is this correct, or is the distribution of products actually just shifted down? Generally, it might be useful to represent the ExoVII cleavage products in graphical form, so that the center and widths of the banding distributions can be more readily evaluated.

6. With respect to the experiments described in Fig.4, there is also an unexpected result in lane #8, that appears to suggest that the presence of the trans TEN domain somehow acts in a dominant negative fashion in the context of the 91-end hTR construct (compare with lane 3). I do not believe the authors comment on this observation or provide any interpretation/explanation for what might be happening here? This should be addressed in the manuscript.

Minor point:

1.The authors choice of the term 'evolutionarily conserved TERT core' to describe the hTERT fragment lacking the TEN domain is somewhat misleading. The TEN domain is also evolutionarily conserved in most species. Moreover, in previous work with the Tetrahymena system, the Collins laboratory and colleagues have used the term 'core RNP' to represent a complex that includes the TEN domain. Therefore, to avoid confusion it would be best if the present study maintained a consistent nomenclature in this regard, perhaps substituting hTERT TEN for 'core' throughout the manuscript.

1st Revision - authors' response 24 January 2014

(please see next page)

Reviewer text is in plain font; *our responses are in italic*.

Referee #1:
Major suggestions:
1. The Finger and Bryan paper entitled "Multiple DNA-binding sites in Tetrahymena telomerase" (NAR, 2008) seems especially pertinent to the current work, even though it relates to telomerase from a different organism. Those authors already showed that the TERT core more than the TEN domain bound DNA. This work could be cited to good advantage in both the Intro and Disc.

The revised text explicitly states the Finger & Bryan 2008 conclusion as well as citing other (often contradictory) studies of TERT-DNA interaction. A large number of TERT-DNA interaction studies have been done, which is why we cite them as described in a detailed review (Wyatt et al 2010).

2. Fig. 1E, 2F and 4C, the Relative Activity values are given to 2 sig figs. What are the uncertainties in these numbers? How many independent expts were performed? Should the values of 1.9 and 3.1 (Fig 4C) be reported instead as 2 and 3? It's fine if repeats of the experiment gave the same trend but the values were enough different that they can't be averaged. But the reader needs an indication of reproducibility.

At least triplicate independent experiments were performed, which we now state in the text. We rounded Relative Activity numbers as suggested and normalized to make them easier to compare.

3. Fig. 8B and p. 17 middle paragraph, "The lengths of protected products generally increased with synthesis across the template." Does this imply that the 5'-tail of the primer does not move relative to TERT? That would be my interpretation. The authors should state theirs. What if they allow translocation? Does the protected region then jump back down, or does it continue to increase? This could give major mechanistic insight.

We more clearly discuss our interpretation of the 5' edge of the protected product not moving with each nucleotide addition: the Discussion and a Figure schematic suggest a constant length of single-stranded DNA protection and increasing length of product-template hybrid. In addition we include data suggesting that the 5' edge of protection can move if repeat synthesis is processive, supporting the conclusion that the product does not have to entirely "loop out" from the enzyme. Implications for telomerase structure and mechanism are described in the revised Discussion.

4. I suggest deletion of the P1 experiments (bottom of p. 11 and Fig. 3B,C) unless the authors perform the compensatory base-pair changes for P1 helix. That is, instead of just mutating the bottom strand, one needs to mutate the top strand by itself or mutate both to give compensatory bp changes. In the absence of this information (which is standard way in the RNA field to establish that a structure exists), one has no confidence that the hTR 18-56 RNA is even forming the P1 helix.

We deleted the trans-template investigation of P1 because the conclusions weren't as meaningful as the rest of the manuscript's content. The role of P1 in template boundary definition is described by citation. The one remaining direct comparison +/- P1 that could be made within our results is not described as an assay of P1 function (Figure 2E).

5. I suggest deletion of the bottom half of p. 20, the speculations about whether template-primer base-pairing accumulates of stays constant during synthesis. The authors' argument 1 is not totally

convincing, because we don't know what else a mismatched primer might be doing that interferes. Their argument 2 is unconvincing, because the accumulating product could be forming a loop.

We retained some revised speculation about the accumulation of product-template duplex because it is a necessary framework for interpreting the nuclease protection results. The revised text gives more detailed justification including rationale for why this lengthening of the product-template hybrid may be a vertebrate telomerase distinction from ciliate and yeast enzymes.

Minor point: On the Fig. 9 model, it'd be nice to have one final diagram to show reengagement for the next round, or alternatively an arrow from iv back to i. In the title, the hyphenated word "template-hybrid" is nonstandard. Please consider "template-primer duplex."

We have added the arrow to complete the catalytic cycle and now use "primer-template hybrid" or "product-template hybrid" as standard in the polymerase field.

Referee #2: [comments are streamlined slightly for focus on concerns; in the long comments, the main point of emphasis is in bold]
Major concerns:

1. Many of the major conclusions rely on a clever, albeit compromised and fragmented telomerase RNP system, as well as quantitatively small (and in at least one case nonexistent) differences in results between experimental conditions ... **There is frequently no indication of the reproducibility or accuracy of the relative telomerase activity results ...** Addressing this issue will require more than rewording, since the take-home message from the data seems, at this point, unsupported and subjective. The additional facet of this overarching issue of quantitative claims is that several of the most important findings rely on accuracy of values that approach zero. Subtraction of telomerase activity background signal is a highly inaccurate, subjective process even with the most modern methods ... Thus, as is the case with multiple conclusions in this report, reliance on a single experimental approach and small-scale quantitative differences in results between conditions - particularly in an ornate pieced-together and compromised telomerase assay system - makes it quite difficult to draw solid conclusions.

All activity assay panels with product intensity quantified as relative activity (in Figures 2, 3, 4, and S4) now have integer numbers only. Weak product intensities are quantified only as "<1" relative to the "10" assigned to the reference lane for normalization (i.e. activity values less than 10% of the reference are not distinguished). All conclusions described in the text were based on results from at least three independent assays, as now stated in the revised text.

2. ... there is complete reliance on the compromised, pieced-together in vitro systems they also are co-reporting here ... the conditions are far from wild type at the outset, and then walk yet further away as the research progresses. The authors do not provide data verifying that the system they propose to have controlled is really behaving as expected. For example, when P1 nts are provided as part of the template fragment, is it known that P1 actually forms? Furthermore, as an indication of how functional or compromised the trans-piece RNPs are, how "active" are they compared to wild type for the nt-addition activity relied upon in this study? Are TEN and CORE portions of TERT fully folded and functional despite being severed from each other? What have the severe truncations of conserved regions of the hTR RNA potentially caused that we might not yet understand? Overall,

it is very hard to know if, when an additional variable is examined in this ornate in vitro system and found to cause a change, it reflects a physiologically relevant function... it would greatly improve the work to return to wild-type or quasi-wild-type factors in vitro (and ideally to human cells for EMBO) to test the basic "take-home" message(s) from the in vitro experiments, in order to **show that the conclusions are not merely based on artifacts related to the collection of variables introduced by the fragmented system** ...

All of the telomerase product DNA protection experiments shown in the original Figures and most of the product protection experiments shown in the Supplementary Figures used human telomerase holoenzyme assembled in cells. We suspect that by starting the Results with heavy emphasis on the utility of trans-template activity assays and the new in vitro TEN domain trans-complementation method, we created an impression that all of the functional and physical assays described in this work used enzyme from RRL reconstitution of fragmented hTR and fragmented TERT. We have extensively revised the text and Figure panels to improve transparency about which enzyme system was used to make each conclusion. We have also expanded the description of the rationale for using each enzyme system and added direct comparisons of results across enzyme reconstitution methods.

Previous work clearly establishes the physiological enzyme relevance of new insights attained using trans-template and trans-complementing TEN domain reconstitutions (Qi et al 2012, Robart and Collins, 2011). We re-validate the physiologically assembled enzyme parallel for both assays as they are performed in this work. Supplementary Figure S2 (previously S1) uses in vivo enzyme reconstitution to parallel the Figure 1B in vitro enzyme reconstitution demonstrating product synthesis fidelity on a trans-template RNA oligo with the native hTR template sequence. Figure 1F validates the new in vitro reconstitution system for TEN domain trans-complementation by paralleling results from the previous trans-complementation method requiring co-expression of TERT ring, hTR and TEN domain in human cells. We have confidence that the TERT ring RNP folds correctly because it is active (Figure 1F, lane 3) and because by TEN domain trans-complementation it regains the high RAP of full-length TERT (compare lanes 1 and 8). For direct comparison, new Supplementary Figure S1 compares full-length TERT assembled with full-length hTR versus hTRmin used here (hTR lacking the H/ACA RNP assembly domain) versus the even more minimized, field-standard, two-piece hTR system that we and others have rigorously benchmarked as physiologically representative for activity reconstitution (starting with Mitchell and Collins, 2000). Figure 1F lane 1 shows the same full-length TERT and hTRmin reaction as Figure S1 lane 3 (literally the same gel lane), which then can be compared to TERT ring RNP with trans-complementing TEN domain in Figure 1F lane 8. The TEN domain must be functional in the reconstituted RNP with high RAP, but most of the recombinant purified TEN domain we suspect to be in an inactive conformation as described more thoroughly in the revised text.

In the critical demonstration of TERT ring RNP activity stimulation by single-stranded DNA (revised Figure 4C and Supplementary Figure S4), we now show parallel trans-template assays of full-length TERT, TERT ring, and TERT ring + trans-complementing TEN domain reconstituted in RRL along with a trans-template assay of full-length TERT RNP reconstituted in cells and assays using internal hTR template in full-length TERT and TERT ring RNPs reconstituted in vivo. Supplementary Figure S5 (former S2) demonstrates equivalent nuclease protection profiles for products of the full-length TERT holoenzyme assembled in cells versus TERT + hTRmin RNP assembled in RRL.

3. Another major concern is the non-telomeric poly-T 5' ends of primers used throughout the work, as it is centrally important to TEN domain and anchor site conclusions. If one does not provide the biologically relevant 5' telomeric DNA sequence yet relies on conclusions based on absence of TEN

domain and anchor site function using these poly-T 5' ends, it is - at minimum - vexing and, at least for this reviewer, quite disconcerting. **How do poly-T 5' ends influence the conclusions being drawn from the experiments about the TEN domain and anchor site involvement in telomerase mechanism**, etc. Does human TERT TEN domain bind poly-T ssDNA? How about 1-4 repeats of (TTAGGG)? And does it bind telomerase core RNP without or with DNA added? More experiments, discussion of this issue in the text, and increased referencing of TEN and anchor-site literature seems critical to deal with this confounding issue.

Morin (1991) established that 5' primer regions of non-telomeric sequence are comparable or better at stimulating human telomerase holoenzyme activity than primers with 5' telomeric-repeat sequence, with a length-dependence to the stimulation that is matched by our nuclease protection results. Wallweber et al. (2003) rigorously demonstrated for human telomerase holoenzyme that the presence of a 5' single-stranded primer region improved enzyme-primer physical association whether the 5' extension was telomeric or non-telomeric, with a length-dependence matched by our nuclease protection results.

As added data (Supplementary Figure S3), we confirmed that a 5' T-tract extension enhances primer use comparably to or better than a 5' telomeric-repeat sequence in assays of full-length TERT RNP with hTRmin assembled in RRL and full-length TERT RNP with full-length hTR assembled in cells. From the revised text: "we used primer with a single-stranded region of poly-thymidine (T), typically in the 27 nt primer T_{21}-GTTAGG (Figure 1A, right). The use of a 5' T-tract obliges base-pairing of the primer 3' end with the template, rather than internal regions, and it precludes primer multimerization by guanosine quadruplex formation. Previous studies establish that in the single-stranded region of a primer, non-telomeric sequences increase primer extension comparably to or better than a single-stranded telomeric repeat..." Here and elsewhere in the text we cite the Morin (1991) results that also are discussed in response to point 5 below.

In the nuclease protection assay, use of 5' homopolymer T-tract primers is essential for eliminating apparent "protection" that derives from sequence-dependent ExoVII cleavage. ExoVII has sequence bias, as noted in the text, which we have confirmed repeatedly while trying to eliminate it. This ExoVII sequence bias affects the profile of protection of TTAGGG-repeat products. Also, telomeric-repeat products fold to quadruplex structures that are protected from digestion whether bound or not bound to telomerase.

We recapitulated major findings by testing telomeric as well as non-telomeric DNA 5' extensions from the primer-template hybrid. Functional assay: Supplementary Figure S4 directly compares TERT ring RNP activity stimulation by single-stranded 5' primer T-tract versus telomeric-repeat extensions. Physical assay: Supplementary Figure 7 (former S3) shows nuclease-protection results for telomerase product DNA of entirely telomeric-repeat sequence, with the major caveat of ExoVII cleavage inhibition on G-rich sequence stated in the main text.

4. Does the purified TEN domain reported by the authors bind DNA by filter binding or gel shift? This should be tested and reported. Does it co-purify with the telomerase RNP when added to the RRL prior to pull-down? If it affects telomerase activity and is not binding 5' extensions of DNA substrate, it should co-purify with the RNP and data supporting this directly should be provided.

Studies with the purified, bacterially expressed human TERT TEN domain would have dubious physiological significance, because this purified domain alone is not functional for catalytic activation of the TERT ring RNP: trans-complementation is not robust enough for activity detection by direct primer extension if the TEN domain is mixed with TERT core RNP after TERT core RNP synthesis (Figure 1F). Several previous studies cited in the text have reported DNA binding activity

or a lack of DNA binding activity for isolated TEN domain. The domain, like the entire TERT protein, is highly basic; we would expect favorable electrostatic associations with nucleic acid, perhaps particularly with telomeric-repeat oligos that support intermolecular pairing and formation of higher-order structure with concentrated negative charge. Sorting out which in vitro TEN domain nucleic acid interactions are meaningful first requires the ability to benchmark physical properties against functional assays such as those described in our work. Our results steer us away from interpreting autonomous DNA binding activity of an isolated human TERT TEN domain.

To show direct evidence that stable TEN domain association converts the TERT ring RNP to high RAP (versus the alternative hypothesis that a transient interaction converts the TERT ring RNP to high RAP), we added Supplementary Figure S1C showing recovery of reconstituted high-RAP activity in association with the tagged TEN domain. This adds another parallel of the in vitro system to trans-complementation by in vivo reconstitution (Robart and Collins, 2011)

5. Also related to the issue of TERT anchor site DNA interaction, published reports such as those of Wyatt et al, 2007 and 2009 from Tara Beattie's group; Lue and Li, 2007; Moriarty et al, MCB 2005 from Chantal Autexier's lab; Hammond papers from 1997 and 1998 from Tom Cech's group; Bairley et al. 2011 from Kathy Friedman's lab, all seem highly relevant, yet none of these papers were cited. Also, the findings linking human telomerase action to 5'-proximal TTAGGG-related sequences in human patients by Morin (Nature, 1991) should be discussed in relation to the unexpected findings in this work about the anchor site and TEN domain.

The large number of publications that investigate a TERT or telomerase RNP anchor site - much larger than the list above - can't be individually described and cited in this manuscript. The content of the listed papers to 2010 has been covered in a detailed review that we do cite (Wyatt et al 2010). Some additional primary references have been included in the revised text. The Morin 1991 paper was the first to show that 5' primer regions of non-telomeric sequence are comparable or better at stimulating human telomerase activity than primers with 5' telomeric-repeat sequence (see point 3 above). We recapitulate this result for both hTRmin RNP assembled in RRL and holoenzyme RNP assembled in cells, directly comparing T-tract versus telomeric-repeat 5' primer extensions (Supplementary Figure S3).

6. There should be thorough discussion of the data in this work as compared to the data and model reported by Zaug, Podell and Cech, NSMB 2008, including discussion of the relevance of Leucine 14 (as well as testing of L14 mutants), since this residue is apparently conserved in hTERT TEN domain.

The qualitative activity defect induced by the Tetrahymena TERT L14A substitution has not been recapitulated in the human enzyme: mutagenesis of two proposed comparable human TERT TEN-domain leucine residues abolished activity reconstitution (Zaug et al 2008). In our lab's studies of the Tetrahymena TERT L14A variant (Eckert and Collins, 2012), L14A reduces the rate of nucleotide addition specifically at the template 3' end, leading to the appearance of low RAP when comparing TERT L14A RNP product profile to wild-type at a single time point. The Tetrahymena TERT TERT L14A results are fully consistent with our new conclusions about TEN domain function.

7. The logic of the experimental design is not obvious in several instances (and over the course of the 9 figures some experiments seem tangential). Why were nucleotide substitutions within the template attempted when investigating boundary function in Figure 3? Was it expected that the template sequence would have such an effect based on Drosopoulos et al. 2005 and what is the

mechanistic explanation/model the authors have for this? Another example is in Figure 5: why is P1 relevant to tests of TEN domain function on 5' extended primers (and extending them by 21 T residues rather than telomeric TTAGGG sequence)?

We have clarified the logic of experimental design and also the logic to the progression of the experiments. The P1 experiments have been removed for streamlining.

8. The title of the manuscript is not particularly informative; could it be more impactful and focused on the most prominent conclusion or two? As it stands, it suggests that the manuscript is spread too thin with multiple claims, often supported by too few experimental results and controls.

The title has been changed.

Minor concerns that should be addressed:
Why not include wild-type control(s) "across the board" to allow the reader to compare relative activity amongst telomerase RNPs between experiments/figures? Connecting the findings and mini-fragment systems developed here to the cellular condition would help the readership determine the degree to which the impressively and extensively reconfigured telomerases are functional, as well as more precisely what ways they have compromised function.

We have added cross-comparisons of enzymes made with different reconstitution protocols, as described in response to major concern 2 above.

The dependence of telomerase on RNA 5' vs 3' of the template should be discussed, as there are several reports of tests of this that have been performed on ciliate (where the TRE was identified and "accordion" model proposed) and very recently also yeast telomerase RNA in vitro and in vivo.

We do discuss the significance of template-flanking RNA regions for template boundary definition in human versus ciliate and yeast telomerases. The revised text better emphasizes the distinction in template 5' boundary definition mechanisms in human versus ciliate/yeasts, but due to the large number of studies on template boundary definition, we cite a review and only the extra primary references necessary to compare different template boundary definition mechanisms.

Fig 2D: Core TERT + hTRmin in RRL but without TEN is missing. It should be included for comparison.

The requested lane has been included in what is now Figure 1F.

Figure 4D: The model drawn is highly speculative, and furthermore based on very subtle differences in activity in lanes of 4C, as mentioned above. Although they are helpful, should models be (a necessary) part data figures in order for results to be interpreted?

We included telomerase RNA schematics and model panels in several Figures to aid reader discrimination of what the different experiments are testing. The number of different points that are made in our studies will otherwise be hard to keep in mind to integrate in the Discussion.

Citation(s) are needed for the origin and reported dysfunctionality of the hTERT D868A mutation. Also, the text did not state that this Asp mutant should be completely inactive, assuming that is the

case and this is why it was tested as a control. (Not all Asp residues in the RT domain disrupt TERT function as shown by Lingner et al., Science 1997, but hTERT D868A appears to correspond to Sc TERT D670A or D671A; mutations which do cause senescence.)

The citation for the catalytic-dead mutation is now included.

Line 2 of p8; as written, it sounded to me like dGTP and dTTP were BOTH labeled with 32P until I inspected the annotation of the figure.

We have clarified that we use radiolabeled dGTP in text and Figures.

Hyphenation related to compound adjectives should be addressed in many places throughout manuscript: e.g., while they are not in the manuscript, it is more appropriate to include hyphens in phrases like "trans-template RNA", "RNA-template oligonucleotides" etc.

We revised hyphenation throughout the text.

Additional suggestions:

Fig 1B: Quantify D868A abundance vs. WT and report relative levels

D868A TERT has undetectable product synthesis and thus we can't use relative protein expression to calculate relative enzyme specific activity.

Referee #3:
Major comments:

1. It is interesting that linking the PK and CR4/5 hTR fragments functions so well to reconstitute telomerase activity. However, there is not a direct comparison between this novel RNA construct, the full-length hTR, and the commonly employed two-piece PK + CR4/5 domain reconstitution. It would be nice to see a direct evaluation of these reconstitution approaches side by side, both in terms of telomerase assembly efficiency and catalytic activity, to better understand how the authors arrived at a linker of 14 nt to be 'optimal'?

Various linkers separating PK and CR4/5 were tried in initial RNP assembly experiments with the goal of generating a maximal amount of stably assembled RNP. However we did not test linker lengths at single-nucleotide intervals, so we changed wording to avoid giving the impression that precisely 14 nt is a magic number for PK and CR4/5 spacing. We show the requested activity comparison as new Supplementary Figure S1C. The revised text more thoroughly describes why we need to use the single-piece hTRmin rather than the two-piece system we developed previously to minimize RNA misfolding and increase RNP specific activity (Mitchell and Collins, 2000).

2. It is striking that the trans TEN complementation system with bacterially expressed TEN domain and RRL reconstituted telomerase RNP only works when the TEN domain is present during the hTERT translation reaction. The authors provide only one of several possibilities for this important observation; namely, that some component of the complex is mis-folded and the co-folding in the RRL is required to produce activity. What is meant by 'co-folding'? As an alternative, is it possible

that there are post-translational modifications of the TEN domain that are occurring in the RRL which may account for this difference? If this were to be the case, it would be interesting to know what these modifications are and how they might relate to telomerase function in vivo. While answering these questions is clearly beyond the scope of the present manuscript, the authors should spend a bit more time discussing the potential implications of the requirement for a very specific order of addition protocol to recover activity in their system.

The revised manuscript gives more detail on the in vitro system for functional trans-complementation by a bacterially expressed TEN domain. Revised Figure 1F shows that RRL incubation of the TEN domain is not sufficient to give it function; instead the TEN domain must be added before synthesis of the TERT ring (compare Figure 1F lanes 8-9). We agree with the Reviewer that there are numerous implications of Figure 1F results; accordingly, we reorganized original Figures 1-3 to revised Figures 1-2 to improve the emphasis.

3. On a related note, this reviewer is curious what is meant by 'an excess of TEN domain' in the methods section describing the TEN trans complementation approach? Are these complexes assayed for telomerase activity directly in the RRL reaction, or are they immunopurified from the lysates? Either way, it would be interesting to know more about the nature of the TEN interaction with the rest of the complex since it is no longer covalently tethered to the rest of the TERT polypeptide? In other words, are the experiments done in the presence of a large excess of soluble TEN domain, or is a single TEN domain stably interacting with the hTERTΔTEN +minhTR fragments as drawn in the models? A more detailed characterization of the TEN trans-complemented enzyme complex is important information for the reader to better interpret the results of the presented experiments. How would the interpretations of the results change if one were to assume a rapid exchange of the trans TEN domain? These points should be discussed unless there is direct evidence suggesting a stable association of a single TEN domain with the rest of the complex.

Rapid exchange is undetectable because trans-complementation is not robust enough for activity detection by direct primer extension if TEN domain is mixed with TERT core RNP after synthesis in RRL (Figure 1F). To show direct evidence that stable association of recombinant TEN domain converts the TERT ring RNP to high RAP (versus the alternative hypothesis that a transient interaction converts the TERT ring RNP to high RAP), we added Supplementary Figure S1C showing recovery of reconstituted high-RAP activity in association with the tagged TEN domain.

4. I am curious whether having the large MBP tag on the TEN domain, which I believe more than doubles its molecular weight, might have an impact on the ability of TEN to complement in trans under certain sensitized conditions? Is there a practical reason why the tag was not removed for these experiments, which would seem to be preferable? And is there reason to believe that some of the trans TEN complementation results would be effected by the presence of the MBP tag?

We find that the MBP tag is essential for TEN domain folding and/or solubility: removing this tag from the purified TEN domain using gentle TEV protease cleavage was greatly inhibitory for trans-complementation. This is now stated in the text as an unpublished observation. We would speculate that alone, separate from the physiologically co-folded TERT ring and hTR, the human TERT TEN domain does not fold properly and the MBP tag prevents it from converting to an irreversibly inactive fold. Large N-terminal tags on human TERT do not interfere with telomere maintenance by the telomerase holoenzyme; thus the N-terminal MBP tag on the TEN domain would not be expected to impose interference for telomerase activity reconstitution.

5. There are several places in the manuscript where the author's choice of which effects are 'substantial' and which are not seems somewhat arbitrary and would benefit from either: (i) a more statistically rigorous treatment of the data, together with error estimates, so that the reader may better appreciate what effects are statistically significant; or (ii) an explicitly more qualitative conclusion in the text. For example:
a. In Fig.4C, the authors describe the telomerase activity observed in the absence of the PK domain to be low but detectable and quantify this activity as 1.9% of the wild type enzyme. Then in the same panel, lane 7, the authors claim that in the absence of trans TEN domain there is no observable activity in the absence of the PK domain, but the relative activity is now 1.5% of wild type. This would seem to suggest that these activities are within the noise of the measurement, and should not be taken as significant in either case. That said, visual inspection of the gels does suggest the author's conclusions are correct, but there may be an issue with the data treatment protocol. Perhaps the authors can use freely available software from the Herschlag lab (SAFA) to integrate the relative amount of each band present, correct for specific activity differences due to sequence content, and then present the data in graphical form? This may lead to more reliable numbers in situations where activity is very low and close to background.
b. A second example is in Fig. 8A, where the authors present ExoVII cleavage data for the full-length TERT vs. the Core and demonstrate that the TEN domain is not required for protection of ~18nt of ssDNA in the complex. The statement is then made that the products appear 'more evenly spaced' with the Core enzyme compared to the Full-length TERT. Is this correct, or is the distribution of products actually just shifted down? Generally, it might be useful to represent the ExoVII cleavage products in graphical form, so that the center and widths of the banding distributions can be more readily evaluated.

We were unfamiliar with SAFA; we thank the Reviewer for suggesting its use. The critical Fig. 8A (revised Figure 6A) DNA product protection by full-length TERT RNP versus TERT ring RNP is now accompanied by new Figure 6B: the intensity profile of protected products as a function of length. Figure 6B shows a clear peak at 18 nt for protected products of full-length TERT RNP, while TERT ring RNP protected products are spread from ~18 to ~14 nt. Intensity profiles of protected products were added in Supplementary Figures S5 and S6 as well. These additions should improve the general readership's ability to appreciate the nuclease-protection assay results.

Previous Fig. 4C (now Figure 3C) and all other activity assay panels with product intensity quantified as relative activity (in Figures 2, 4, and S4) now have integer numbers only. Weak product intensities are quantified only as "<1" relative to the "10" assigned to the reference lane for normalization (i.e. activity values less than 10% of the reference are not distinguished). All conclusions described in the text were based on results from at least three independent assays, as now stated in the revised text.

6. With respect to the experiments described in Fig.4, there is also an unexpected result in lane #8, that appears to suggest that the presence of the trans TEN domain somehow acts in a dominant negative fashion in the context of the 91-end hTR construct (compare with lane 3). I do not believe the authors comment on this observation or provide any interpretation/explanation for what might be happening here? This should be addressed in the manuscript.

These results are revised Figure 3C, lanes 3 and 8. We have boxed them in red to draw attention to the comparison. The revised Results text states "the presence of P2a.1/P2a was more critical for activity in the presence versus absence of TEN domain" and includes our interpretation "vertebrate-

extended P2a.1/P2a influences coupling of the TEN domain and TERT ring." The Discussion integrates this into the new perspective on catalytic cycle mechanism.

Minor point: The authors choice of the term 'evolutionarily conserved TERT core' to describe the hTERT fragment lacking the TEN domain is somewhat misleading. The TEN domain is also evolutionarily conserved in most species. Moreover, in previous work with the Tetrahymena system, the Collins laboratory and colleagues have used the term 'core RNP' to represent a complex that includes the TEN domain. Therefore, to avoid confusion it would be best if the present study maintained a consistent nomenclature in this regard, perhaps substituting hTERTΔTEN for 'core' throughout the manuscript.

The revised manuscript uses "TERT ring" to describe the structural core of TERT, based on its folding as a three-domain ring around the active site.

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Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by all three original referees (see comments below), and I am happy to inform you that there are no further objections, and all referees now recommend publication in The EMBO Journal.

Referee 2 still has some comments that you may want to address by minor changes to the manuscript text. Therefore, please consider these suggestion and send us a modified text document simply via email.

Referee #1

The revised paper by Wu and Collins has responded well to the referees' comments, and it is my opinion that the paper is now suitable for publication. Allow me to reiterate why this contribution is a strong one for EMBO J. First, it represents a landmark contribution regarding human telomerase enzymatic mechanism that is essential for the telomerase field. One highlight is a rebuttal of the model that the TEN domain contains an anchor site binding single-stranded DNA and replacing this model with a new one in which the TEN domain allows active-site use of the short primer-template hybrids characteristic of telomerase. Furthermore, the paper reports the first direct footprinting assay of which portion of the DNA primer is protected from nuclease by telomerase, with the major conclusions that the pattern is the same plus-or-minus TEN, and that during extension the DNA appears to slide along the enzyme rather than looping out from a fixed anchor point. These are technically challenging experiments, and few workers would be capable of executing them with the precision seen here.
This work will also be of broad interest to the polymerase and reverse transcriptase fields. A fundamental question has been: how does an RT become adapted during evolution for multiple repeat synthesis (telomerase) vs. tracking along a retroviral RNA template? This work now highlights the telomerase-specific TEN domain as a major contributor to this adaptation.

Referee #2

The manuscript is almost completely rewritten and figures reordered, with some results and data removed as well as some new experiments added. These changes do focus the work some more and generally help show that the results are supportive of - or at least consistent with - the authors' conclusions.

Due to all of the alterations, the manuscript is almost like a new submission. The authors' additional effort has somewhat improved the impact of the work. Nevertheless, the general increase in controls and improved quantification accuracy (e.g. by reducing the claimed degree of precision) is notably better. As for reproducibility, it would have been better to see standard error stated numerically for values listed in figures rather than relying on an N of 1 for single integers shown and accompanied only by statements in the text about experiments being done several times. As a result the reader must still just assume data shown were all "representative."

The authors have addressed some of the concerns raised earlier. For example, they have added some benchmarking to wild-type telomerase to make it clearer that their results are unlikely to be simply artifacts of the highly mutated RNAs being employed, in trans TEN protein fragment (which the authors say has a small active fraction of molecules), etc. Clearly the authors remain the best judge of how directly relevant their results are to the in vivo condition.

The words "telomerase adaptation" in the last sentence of the abstract were unclear. Furthermore, the statement that the findings reported in this paper "change the paradigm" with respect to this "adaptation" seemed like an overstatement. It also conflicts with how the Introduction is written,

where the paradigm is spelled out as consistent with the authors results, while the case for "dogma" (top of p. 6)
proposing the TEN domain binds DNA is only supported by the authors' citing a select few papers (while the authors say in the response to my question 5 that the literature for TEN-DNA binding is "much larger" than the seven I mentioned and "can't be individually described and cited").

The hypotheses and unique perspectives of the authors - now even more evident in the Introduction and Discussion - can be considered to add novelty to this report and publishing would allow the field to consider and test the authors' interesting ideas and claims.

Remaining issues:

The first sentence of the Results states that "We sought mutagenesis-independent approaches..." It seems the authors must be referring to the TERT subunit (not the RNA), and specifically sequence substitution changes (?) here, as clearly the RNAs most heavily utilized are quite mutated by truncation and the TERT protein is also mutated by breaking it into separate fragments. I see no need to avoid "mutagenesis" but rather it should be embraced with careful attention to caveats it can introduce.

The penultimate sentence of the paragraph in the middle of p. 8 is confusing as it is not obvious how Qi et al. 2012 used a "heterologous template" or how it was different from the current study. What does "heterologous template" really mean in this context? Presumably this should be easy to fix by stating things more precisely.

There are places where citations are still inappropriate, such as the following examples:

1. 4th line from bottom of p. 4, Podlevsky et al 2008 is cited when really the original Chen, Blasco and Greider, Cell 2000 paper warrants referencing for "vertebrate TER secondary structure established by phylogenetic comparison..."

2. on the bottom of p. 10, the Podlevsky et al., 2008 TER database paper is cited for the first identification of the yeast template boundary element helix ("template- flanking stem"). This is really inappropriate referencing. Tzfati...Blackburn, Science 2000 is correct.

Referee #3

In their substantially revised manuscript, Wu and Collins have done an excellent job addressing the previous round of reviewer comments. Inherent in their experimental approach is the assumption that by artificially separating parts of the telomerase complex, and adding them back together in a systematic fashion, useful information may be attained relating to the function of the unmodified telomerase enzyme. While this approach certainly comes with caveats, as raised in the previous reviews, it is certainly not out of the ordinary in mechanistic biochemical studies - particularly on an enzyme as difficult to reconstitute as telomerase. Indeed, in the absence of a high-resolution structure of the telomerase enzyme bound to its DNA substrate, biochemical dissections such as those described in the present study are a critical means to advance our knowledge of the structure-function relationship of this important enzyme. I recommend the paper be accepted and published in EMBO in its current form.

