THE EMBO JOURNAL

Manuscript EMBO-2013-85448

RNA connectivity requirements between conserved elements in the core of the yeast telomerase RNP

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Review timeline:

Submission date:	23 April 2013
Preliminary Editorial Decision:	23 May 2013
Editorial Decision:	07 June 2013
Revision received:	24 August 2013
Pre-acceptance letter:	16 September 2013
Additional correspondence:	19 September 2013
Accepted:	23 September 2013

Editor: Hartmut Vodermaier

Transaction Report:

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

1st Editorial Decision (preliminary) 23 May 2013

Thank you again for submitting your manuscript on telomerase RNA connectivity for consideration by The EMBO Journal. We have so far received two sets of comments and are currently waiting for a (slightly delayed) third report. Since I will be away from the office for the next ten days, I have now carefully look through the two reports already at hand, and decided to contact you at this point with a preliminary decision. Both referees 1 and 2 are overall positive and would in principle support publication, however pending adequate revision of a number of concerns with both the experimental analysis and aspects of presentations. As most of these points appear to be readily addressable, we would be interested in considering a revised version of this manuscript further for publication. I would thus like to invite you to start revising the manuscript according to the referees' comments and suggestions. I nevertheless have to stress that this is remains a preliminary decision at this stage, and thus still subject to change should the last, missing report raise serious additional concerns. Once we will have received the last report, we will contact you to transmit it and finalize this editorial decision.

I should add that it is EMBO Journal policy to consider only one single round of major revision, and that it is therefore essential to satisfactorily address all the main points at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html).

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:

In the manuscript "RNA connectivity requirements between conserved elements in the core of the yeast telomerase RNP," Zappulla and colleagues use detailed mutagenesis of the telomerase RNA to examine the flexibility of the TLC1 RNA component of telomerase in enzyme function both in vivo and in vitro. Previous work from the Cech and Zappulla groups identified a minimal TLC1 RNA (micro-T) capable of supporting telomerase activity in vitro in conjunction with the catalytic protein Est2. Micro-T contains four recognizable structural elements that are highly conserved among all telomerase RNAs both in nature and in relative position. In this manuscript, the authors examine whether this core sequence can accommodate either strand breaks (in the form of circular permutations) or alterations in the linker sequences that join each core element. Using this strategy, the authors suggest that the order of elements is critical to telomerase function and define an "Area of Required Connectivity (ARC)", a region encompassing all of the structural elements that must be physically tethered through either backbone or secondary structure interactions. All of the mutations are also created in a larger RNA construct (mini-T) that contains binding sites for accessory factors and allows telomerase function in vivo. The results obtained in vitro and in vivo are perfectly correlated across assays in both RNA constructs, increasing confidence in the conclusions. While this work is in a sense descriptive (the authors speculate about, but do not test, the essential function of the ARC), the work is important in defining further the remarkable flexibility in RNP function and lays the groundwork for future mechanistic studies. The manuscript is written very clearly and the diagrammatic representations are helpful with a few minor suggestions as given below. The biggest omission is detailed quantification for some of the experiments.

Major comments:

1. The inclusion of quantified RNA levels and the range of values obtained in two independent experiments (Figure 7) should be extended throughout the figures of the paper. Otherwise, there is no indication of the reproducibility of the results. The same is true for the relative activity obtained in the in vitro assays. The actual values and ranges obtained should be given either in the figure or within the figure legend.

2. It is unclear how the authors identified the precursor form of the Mini-T RNAs indicated by an open triangle in Figures 4, 5, and 7. What is this precursor form? Was it characterized in a previous publication? If so, that manuscript should be clearly referenced. In Figure 5, some of these smeary forms appear to persist in cells in which there is no mature form RNA (lanes 12, 13, 14, 16). There are also several other bands visible that run at a size intermediate to that indicated as mature or precursor RNA (Figure 5, in particular). This issue should be clarified in the text.

3. The authors use one RNA mutant construct to conclude that the core-enclosing helix is important for telomerase activity for reasons beyond providing connectivity (Figure 8). This conclusion would be greatly strengthened by showing that the same result is obtained when a second cp allele is utilized (for example, one of the alleles lacking connectivity in J3). Otherwise, it is difficult to rule out an effect on secondary structure unique to that single allele.

4. The manuscript would benefit from a more clear definition of the ARC. For example, on page 25, the authors write that, "...we find that the ARC in S. cerevisiae is the area of the core that does not

tolerate breaks in the RNA backbone." But, in fact, the original mini-T has a break within the backbone within the ARC in the context of the core-enclosing helix and other breaks are tolerated within the structural elements, as the authors demonstrate. To avoid confusion, it may be helpful to define the ARC as the region of the RNA from the pseudoknot through to the template that requires connection either covalently through the RNA backbone or through stable RNA secondary structure as afforded by the pseudoknot, the core-enclosing helix, or the template boundary element.

Minor comments:

1. It is misleading in the introduction to call the Sm protein complex "non-essential" since cells lacking the complex do not survive. The text should be clarified to indicate that the Sm complex in not essential for telomere maintenance.

2. It would be useful to the reader to indicate in the text and/or figure legends that the yeast strains utilized for complementation are lacking RAD52.

3. The statement on page 22 that repeat addition processivity (RAP) "is known to be lacking in the S. cerevisiae telomerase core enzyme in vitro" should be clarified to be true on standard yeast telomeric templates. The Lue lab has shown that yeast telomerase can undergo RAP on other types of templates (Nucleic Acids Res. 2004 32(1):93-101. Yeast telomerase is capable of limited repeat addition processivity).

4. The legend for Figure 2 explains "the dotted box," but there are actually two dotted boxes.

5. In general, the gray lettering is difficult to see. Although I appreciate the authors' great efforts to distinguish mutants by function, the figures are actually very clear as constructed and I don't think that the gray lettering is usually required.

6. I don't particularly like the juxtaposition of the outline of a 3D structure for TERT and the diagrammatic secondary structure of the RNA in the model figure. The discussion is quite clear and is not aided by this diagram. The aspect of the figure that shows the ARC is helpful.

7. Several times, the authors refer to the "primary sequence" of the RNA. This is incorrect terminology. They should refer either to the sequence or to the primary structure.

Referee #2:

The manuscript by Mellord and colleagues defines an area of required connectivity within the core of the yeast telomerase RNA to understand conserved element coordination in telomerase mechanism. Importantly, the ARC is an evolutionarily conserved feature of telomerase RNAs.

Minor comments

While the shorter telomere lengths of some viable mutants are consistent with reduced telomerase activity of some of the mutants (J1 insertion/deletion Fig 6C, 7C; J4i5), this is not the situation for other mutants (cpJ3; cpTBE, cpPKc; Fig 3B, 4C). What are the authors speculation regarding the shorter telomeres of these mutants, especially cpJ3 with robust activity? Moreover, one nonviable mutant with no telomerase activity (cpj4; Fig. 3B, 4C) has telomere lengths comparable to MiniT. Can the authors suggest an explanation?

Due to the different sequences within the Mini-T and Micro-T, the breakpoints within the different elements are different when made using the Mini-T or Micro-T. Although the results using either RNA are very similar, is it worth to highlight this difference, perhaps by naming the RNAs differently?

The altered pausing observed for J1s (Fig. 6C) in MiniT, but not Micro-T, is intriguing. Can the authors underline the bases that could form the potential G-C base pairs in the Supplemental Fig 1?

1st Editorial Decision 7 June 2013

We have now received the outstanding third set of comments on your manuscript on telomerase RNA connectivity, which I am copying below for your information. As you will see, also referee 3 is in principle supportive of publication, but raises a number of technical concerns that will need to be satisfactorily addressed before publication.
Therefore, please take these additional points into account when revising the manuscript, and carefully respond to all three referee reports in your point-by-point letter upon resubmission.

With this third report, I confirm my initially preliminary decision on your manuscript.
Therefore, please follow the instructions and resubmission link detailed in my previous letter for preparing and uploading a revised version of this manuscript. Should you have any additional questions in this regard, please do not hesitate to contact me.

Referee 3:

Overall summary:
The study presented by Mefford, et al. probes the connectivity and positioning requirements between various conserved secondary structural elements in yeast telomerase RNA. The main conclusion of the work is that there is an area of required connectivity (ARC) between the pseudoknot (PK), the core-enclosing helix (CEH), the template boundary element (TBE) and the template. This was tested in vitro with the Micro-T RNA system (encoding a minimal RNA required for in vitro activity) and in vitro and in vivo using the Mini-T RNA system (also encoding a smaller yeast RNA with the minimal requirements for in vivo activity) using circular permutants. While there are concerns about extra RNA sequences at the 5' and 3' ends of the RNA obtained from T7 RNA polymerase transcription as well as extra RNA sequences at the 3' ends of the RNA in the case of Micro-T RNAs used in vivo, the broad agreement of their results across multiple systems is a strong argument in favor of their model.
The authors went on to test the effects of insertions, deletions, and substitutions in the junctions between these secondary structure elements. The main conclusion of these experiments was that there is a requirement for at least two nucleotides between the PK and the CEH for in vitro and in vivo telomerase activity. Furthermore, consistent with their results with circular permutants, the junction between the template and the PK is almost completely dispensable for telomerase activity. The authors concluded by testing whether the role of the CEH was to simply connect this region of the RNA together, or if there was a strict requirement for a helix at this position. They discovered that indeed a helix was required at this position for telomerase activity.
This study presents novel results of great interest to the telomerase field. The work is of broad interest to biology in that it addresses an important and poorly understood biomolecule, and uses interesting techniques that could be broadly applicable to other RNA studies. However, the paper should address concerns about the additional sequences incorporated into the RNA ends in these circular permutant studies. For instance, the use of chemically-synthesized RNAs generated by splint- ligation for a small subset of these CP mutants could dispel any concern about additional nucleotides at the 5' and 3' ends. In addition, while the authors carefully designed RNA sequences to prevent large folding defects using secondary structure prediction software, it is known that this software is not 100% accurate in determining RNA secondary structures and that the pseudoknot in particular may be highly sensitive to folding defects due to RNA mutations. The paper may also need to address these concerns, possibly through RNase or chemical probing on a small subset of RNAs used in the study that present large-scale changes in telomerase RNA sequence.

Point-by-Point Summary:
1) By re-arranging the positions of the three major secondary structure elements (PK, CEH, TBE) in S. cerevisiae micro-T RNA, the authors determined that the conserved position of these factors are functionally important. This is a novel, if rather unsurprising, result.

2) The authors used circular permutants to define an area of required connectivity (ARC) between the PK, CEH, TBE, and template. Importantly, the region 3' of the template, known as J3, had no

requirement for connectivity.

3) The functional significance of this connectivity was confirmed in vivo using the Mini-T telomerase RNA system.

4) The authors went on to define the contribution of deletions, insertions, and substitutions in junction residues.
a. The junction between the PK and CEH was found to have a conserved length but not sequence requirement. Deletion of nucleotides had a severe effect both in vitro and in vivo. Insertion of nucleotides displayed a strong defect in vitro, though not to the extent of deletions in this region and cells did not appear to senesce in vivo.
b. Insertions and deletions to the junction between the CEH and TBE was similarly found to have defects in vitro, but not in vivo.
c. The J3 junction was remarkably tolerant of both large deletions and insertions.

5) The authors tested the requirement for a helix at the CEH. They discovered that removal of the helix while conserving the length between the PK and TBE completely abolished Micro-T activity in vitro. They established that a helix is necessary in this region.

6) The authors discovered that the Sm binding site can be moved on telomerase RNA and still have the same effect on RNA stabilization, suggesting that the position of the Sm site with regards to the core is not important.

Concerns:

1) Due to the use of run-off T7 transcription to generate these RNAs, the ends of these circular permuted RNAs do not resemble the clear cut ends represented in Figure 2. Instead these ends likely have several G residues at the 5' end and several U residues at the 3' end. Thus an alternative explanation for the loss of function observed in a subset of the CP mutants is that these positions do not tolerate large additions of nucleotides at these positions.

2) This issue can also extend to the Mini-T results obtained in vivo. I don't know a lot about in vivo RNA processing in yeast, but is it possible that these RNAs do not have well-defined 3' ends, and that the addition of large RNA sequences in these CP mutants may actually be having a larger effect than the loss of connectivity alone?
Certainly the addition of a bulky Sm-binding site at the 3' end of these constructs could be having very large steric effects beyond simply breaking the connectivity at this position. Nevertheless, it should be noted that the congruency between the in vitro and in vivo data is a good argument in favor of connectivity alone. Still, I think alternative possibilities should be mentioned in the text. The use of possibly just one splint-ligated, chemically synthesized RNA (probably to test connectivity in J4) to test the in vitro results may also be a good idea.

3) My understanding is that while mFold is a useful tool, it is not 100% accurate in determining RNA folding in real-life conditions. I don't think we can completely disregard the possibility that some of these mutations are having an effect on RNA folding in the absence of an alternative method, such as chemical or RNase probing.

4) Often times northern blots indicate a substantially reduced RNA accumulation vs. WT mini-T. Can we have an explicit explanation of approximately what levels of Mini-T are required to not show a senescence phenotype? For instance, in Figure 4B, mini-T off of a CEN plasmid shows 15% the levels observed from Mini-T off a 2u plasmid. Were these tested for a senescence phenotype? That would be a strong argument that the 15% observed from a cp-J2 mutant was not the cause for senescence, but rather the specific mutations themselves.

5) In Figure 4C, many of the mutations that showed a senescence phenotype nevertheless appear to have reasonably long telomeres after 50 generations. I assume the take-home here is that these mutants show telomere shortening at 50 generations that it takes 300 generations to reach in other backgrounds. Perhaps a more informative comparison is between all of the telomerase RNA alleles at 50 generations? In general, perhaps a clearer explanation of what this gel tells us for people in a

non-yeast-telomerase background would be useful.

6) In Figure 4A, we see that the cpPKa mutation that was found to be deleterious in vitro has no effect in vivo. Why would this be the case? Is the slight residual activity observed in vitro good enough in vivo?

7) I'd be interested to know if there is a sequence-specific requirement in the CEH. It clearly needs to be a helix, as shown in Figure 8. However, does any helix at this region suffice? Or are there specific sequence requirements? This would be useful in determining, for instance, if the CEH is a protein binding site.

8) A very strong possibility for the defects observed when deleting the 2 nt junction between the CEH and the PK is that the loss of this junction prevents PK folding. With no intervening sequences between the PK and the CEH, it may be that PK folding is being sterically blocked by the CEH. The possibility of the junction influencing RNA folding was covered in the discussion section, however this seems like a very obvious explanation that was not explicitly stated.

9) The discussion mentions the near-universal conservation of core-enclosing helices throughout telomerase RNAs. But in human telomerase RNA, the CEH and the TBE are fused into a single entity, making it difficult to tease apart their relative contributions. Does the human system "count" as an example of the conservation of CEHs?

1st Revision - authors' response 24 August 2013

Referee #1:

Major comments:

1. The inclusion of quantified RNA levels and the range of values obtained in two independent experiments (Figure 7) should be extended throughout the figures of the paper. Otherwise, there is no indication of the reproducibility of the results. The same is true for the relative activity obtained in the in vitro assays. The actual values and ranges obtained should be given either in the figure or within the figure legend.

We certainly agree that reproducibility, accuracy, and precision are important and we have maximized these in the research described in this manuscript. Most importantly, the degree of quantitative precision we have is sufficient for the conclusions we draw from the data in this manuscript, which are based on fundamental, qualitative differences, not quantitative differences between mutants that we have characterized *in vitro* and *in vivo*. We elaborate on this here:

1. Reproducibility. In the initially submitted manuscript, we had mentioned when describing most of the individual experiments that all results had been done at least in duplicate. However, it was not explicitly stated in every case, and this may have concerned the reviewer. We have now modified the manuscript to be sure that the N being two or more is stated for each result, either in a figure legend or Methods. As for the extent of overall reproducibility in the research reported in this manuscript, it is very important to also note that many mutants we have examined are related and comprise a group of alleles that all provide evidence for the same conclusion. Consequently, biological accuracy is much greater than reflected by an N of ≥2 for a given individual allele. And, of course, nearly all mutants tested *in vitro* were also tested *in vivo*, with perfect correlation of *in vitro* activity and *in vivo* function, or lack thereof.

2. Quantitation. High precision is not required to support our conclusions in this manuscript given they are all based on qualitatively clear differences between mutants (e.g., presence or absence of telomerase function). Nevertheless, we have redoubled our efforts to quantify the data, as we summarize below.

Northern blots: The reviewer points to the northern blot in Figure 7B as the benchmark for how quantitation should be performed. However, given the RNA constructs in the

experiment in Figure 7B have only a few nucleotides deleted or substituted compared to wild type, quantifying RNA abundance for these alleles was rather straightforward, particularly when compared to situation in which RNAs have entirely different 5′ and 3′ ends due to circular permutation (i.e., the northern blots shown in Fig. 4B and 5B). These circular permutants have different relative amounts of processed and precursor RNA forms, relatively low abundance, and the associated precursors span a wide range of lengths (more on this is below in the next section). These conditions make it highly challenging to quantify these RNA forms with high biological and technical accuracy. For example, background subtraction is very difficult using any existing software, when the telomerase RNA forms span so much of the lane. Additionally, it remains possible that precursor forms could provide some function *in vivo*, but which, if any, are active is not known. Nevertheless, to address the reviewer's request, we now report quantified RNA abundance for all forms of the telomerase RNA, as well as specifically the processed form. Graphs of the quantified data are in Supplementary Figure 4. These graphs (with error bars) now convey more detail as to the degree of accuracy and precision in our measurements and make it even clearer that our conclusions are sound. For example, the data show that all of the circular permutant RNAs with intact Sm sites are detectable at levels that are known to be sufficient to support telomere maintenance, and therefore for those cp mutants that lead to senescence, their abundance is not the cause of the phenotype.

Telomerase assays: We had already quantified all of the telomerase activity assay results and conveyed this information as a binning system in the initial manuscript ("–", no detectable telomerase activity; "+", less than 50% activity; "++", greater than 50% activity; see Figures 1B, 2B, 3B, 6B, 6C and 8). We felt this was sufficient to support our major conclusions, which only necessitate knowing if a particular telomerase RNA allele supports any detectable telomerase activity. It is important to mention that quantifying telomerase assays and getting very accurate, precise numbers (i.e., detecting relative differences less than two-fold) is very challenging due to (1) differences between rabbit reticulocyte-synthesized and assembled telomerase ribonucleoprotein complex preparations, (2) batch-to-batch variations in the background signal from [α-^{32}P]-dGTP or [α-^{32}P]-dTTP, and (3) limitations in the parameters for quantitation software (e.g., even GE's latest version of Imagequant). Our experience originally developing and employing the reconstituted *S. cerevisiae* telomerase assay (Zappulla *et al*., 2005; Zappulla *et al.*, 2009; Qiao *et al*, 2008) has led us to the conclusion that, given the challenges listed above, it can become futile to try to characterize small differences in activity. Thus, although we continue to quantify our results as accurately as possible, we tend to pursue the most qualitatively obvious phenotypes.

2. It is unclear how the authors identified the precursor form of the Mini-T RNAs indicated by an open triangle in Figures 4, 5, and 7. What is this precursor form? Was it characterized in a previous publication? If so, that manuscript should be clearly referenced. In Figure 5, some of these smeary forms appear to persist in cells in which there is no mature form RNA (lanes 12, 13, 14, 16). There are also several other bands visible that run at a size intermediate to that indicated as mature or precursor RNA (Figure 5, in particular). This issue should be clarified in the text.

Briefly, the processing of TLC1 is not well-understood, but the Cech, Wellinger, and Vasiljeva labs — in several publications since 1997 — have shown that TLC1 has a plethora of longer "precursor" forms due to multiple 3′ ends generated via Nrd1/Nab3 transcriptional termination, polyadenylation, and Sm-mediated exosome trimming. It is also important to note that the blots of polyacrylamide-urea gels blots we perform resolve processed Mini-T and its additional forms (~450–1000 nts) far better than wild-type TLC1 and its precursors (~1150–1300 nts). As stated above, it is not clear whether any of the larger or shorter forms of the RNA may be functional. Thus, we now include quantitation of the mature 460-nt band (a conservative estimate of telomerase RNA abundance, on which we base our conclusions) as well as the total amount of TLC1 RNA (including all of the other forms of the RNA) in Supplemental Figure 4.

We did not characterize the nature of the precursor forms of the RNA (such as by performing an oligo-dT immunopurification to determine which forms of the RNA are polyadenylated), as this was beyond the scope of this manuscript. However, in data not included in the manuscript, we did

examine RNA from saturated cultures (where it is known that precursor forms are decreased), and found that the amount of the larger smeary bands was substantially decreased, consistent with these being precursors. Furthermore, it is clear that the signal we detect represent forms of TLC1, since it is visibly absent in the *tlc1*Δ samples (see northern blots in Figs. 4B, 5B and 7B). In this work, we focused on the functionality of the telomerase RNA alleles we generated and simply point out the that there may be effects of the circular permutations on the efficiency of telomerase RNA biogenesis, given that we have changed the context of both the 5′ and 3′ ends. This is an interesting observation that could provide further insight into TLC1 biogenesis. We have now altered the text to more clearly reference the literature that support the conclusion that these longer TLC1 forms are precursors.

From the experiment shown in Figure 5, it appears some precursor forms exist when there is no mature RNA due to deletion of the Sm site; however, it is the lack of mature-length RNA that is significant to the conclusion that the repositioned Sm sites are functional. We propose that the detectable RNA signal in the Sm- mutants are precursor transcripts detectable in these actively cycling cells (note, the Sm site has recently been shown to be important for exosome trimming of the poly-A tail (Coy *et al*., 2013) and, without a functional Sm site, polyadenylated RNAs may ultimately be degraded by the exosome).

3. The authors use one RNA mutant construct to conclude that the core-enclosing helix is important for telomerase activity for reasons beyond providing connectivity (Figure 8). This conclusion would be greatly strengthened by showing that the same result is obtained when a second cp allele is utilized (for example, one of the alleles lacking connectivity in J3). Otherwise, it is difficult to rule out an effect on secondary structure unique to that single allele.

We agree with the reviewer and have generated three additional alleles to further test if the core-enclosing helix is a required element in yeast telomerase (cpJ3aDCEH, cpJ3bDCEH, and cpPKcDCEH). Consistent with the original conclusion that the CEH is essential, we find that none of these additional mutants exhibit telomerase activity. Thus, the manuscript now demonstrates that the core-enclosing helix is required in four different constructs.

4. The manuscript would benefit from a more clear definition of the ARC. For example, on page 25, the authors write that, "...we find that the ARC in S. cerevisiae is the area of the core that does not tolerate breaks in the RNA backbone." But, in fact, the original mini-T has a break within the backbone within the ARC in the context of the core-enclosing helix and other breaks are tolerated within the structural elements, as the authors demonstrate. To avoid confusion, it may be helpful to define the ARC as the region of the RNA from the pseudoknot through to the template that requires connection either covalently through the RNA backbone or through stable RNA secondary structure as afforded by the pseudoknot, the core-enclosing helix, or the template boundary element.

The reviewer makes a valid point. We have modified our description of the ARC on pages 21 and 25 of the Discussion to clarify that the ARC needs to be connected via covalent connections through the RNA backbone or else by noncovalent base-pairing bonds associated with secondary structure.

Minor comments:

1. It is misleading in the introduction to call the Sm protein complex "non-essential" since cells lacking the complex do not survive. The text should be clarified to indicate that the Sm complex in not essential for telomere maintenance.

This is another good point, and we have modified the Introduction accordingly.

2. It would be useful to the reader to indicate in the text and/or figure legends that the yeast strains utilized for complementation are lacking RAD52.

While we provided the strain genotype in the Materials and Methods section, we have added an additional sentence in the Results section to make this fact clearer to the readers.

3. The statement on page 22 that repeat addition processivity (RAP) "is known to be lacking in the S. cerevisiae telomerase core enzyme in vitro" should be clarified to be true on standard yeast

telomeric templates. The Lue lab has shown that yeast telomerase can undergo RAP on other types of templates (Nucleic Acids Res. 2004 32(1):93-101. Yeast telomerase is capable of limited repeat addition processivity).

To address this issue, we have modified the sentence in question to more accurately reflect that we do not observe repeat addition processivity in our reconstituted *in vitro* assay using a standard substrate oligo.

4. The legend for Figure 2 explains "the dotted box," but there are actually two dotted boxes.

We thank the reviewer for noting our omission. We have corrected the figure legend.

5. In general, the gray lettering is difficult to see. Although I appreciate the authors' great efforts to distinguish mutants by function, the figures are actually very clear as constructed and I don't think that the gray lettering is usually required.

As per the reviewer's advice, we have changed the gray lettering to black in the labeling of mutants in Figures 4, 5, and 7.

6. I don't particularly like the juxtaposition of the outline of a 3D structure for TERT and the diagrammatic secondary structure of the RNA in the model figure. The discussion is quite clear and is not aided by this diagram. The aspect of the figure that shows the ARC is helpful.

We have replaced the outline with smooth lines to more generically represent TERT structure and bring the level of resolution closer to the less well-understood RNA structure.

7. Several times, the authors refer to the "primary sequence" of the RNA. This is incorrect terminology. They should refer either to the sequence or to the primary structure.

We thank the reviewer for pointing out this redundant use of language. We have corrected this issue.

Referee #2:

Minor comments

While the shorter telomere lengths of some viable mutants are consistent with reduced telomerase activity of some of the mutants (J1 insertion/deletion Fig 6C, 7C; J4i5), this is not the situation for other mutants (cpJ3; cpTBE, cpPKc; Fig 3B, 4C). What are the authors speculation regarding the shorter telomeres of these mutants, especially cpJ3 with robust activity?

It is true that cpTBE, cpPKc, and cpJ3 have robust telomerase activity *in vitro* but have telomeres that are shorter than wild-type Mini-T *in vivo*. We believe that this most likely results from the decreased RNA abundance in these circular permutants *in vivo* (shown in Fig. 4B, lanes 6, 8, and 13). Additionally, it is possible that these circular permutations cause defects in holoenzyme coordination (i.e., decreasing the function of Est1 or Ku because the core between these protein binding arms is no longer covalently connected).

Moreover, one nonviable mutant with no telomerase activity (cpj4; Fig. 3B, 4C) has telomere lengths comparable to MiniT. Can the authors suggest an explanation?

While it does appear that Mini-T cpJ4 has telomere lengths similar to the very short telomeres of wild-type Mini-T (Fig. 4C, lanes 5 and 9), the nonviable cpJ4 telomeres shown are from 50 generations since cpJ4 is a senscent alelle. For comparison, the telomeres of *tlc1*D at 50 generations also appear longer than the wild-type Mini-T telomeres at 350 generations (Fig. 4C, lanes 3 and 5). The main reason for this experiment was simply to test if the telomeres in telomerase-deficient ARC mutants were undergoing shortening, consistent with their senescent phenotype, and indeed they were.

Due to the different sequences within the Mini-T and Micro-T, the breakpoints within the different elements are different when made using the Mini-T or Micro-T. Although the results using either RNA are very similar, is it worth to highlight this difference, perhaps by naming the RNAs differently?

We tried to use the same breakpoint for circular permutations in both Mini-T and Micro-T. In fact, the breakpoints are at the equivalent nucleotide in 6 of the cp mutants (cpJ1a, cpJ1b, cpJ2, cpPKa, cpPKc, cpJ4). For the other 2 cp mutants (cpTBE and cpJ3), the break points are slightly different. Altering the exact location of the circular permutation was motivated by *Mfold* RNA folding predictions, which suggested potential misfolding when the ends were placed at exactly the same position in Mini-T as they were in Micro-T (specifically, the repositioned Sm site was predicted to misfold). However, as the referee also points out, all of the *in vitro* and *in vivo* results correlate strikingly well.

These details of the subtle differences motivated us to provide the exact sequence for each mutant in Supplemental Figure 1, as well as including Supplemental Figure 2 with a nucleotide-resolution image of Micro-T(460), so that readers can clearly see the precise location of the new break points. However, we feel that using the same nomenclature for mutants in both Micro-T and Mini-T remains justified (given that 6 out of 8 alleles are in fact at the equivalent position) and helps to highlight the congruency between equivalent mutations in both systems.

The altered pausing observed for J1s (Fig. 6C) in MiniT, but not Micro-T, is intriguing. Can the authors underline the bases that could form the potential G-C base pairs in the Supplemental Fig 1?

We agree that this is an interesting finding that is consistent with, and extends, the template boundary definition by a helix in yeast, as previously described (Seto et al., 2003; Box et al., 2008). We have included our proposed extended TBE pairing in Supplemental Figure 1.

Referee # 3

1) Due to the use of run-off T7 transcription to generate these RNAs, the ends of these circular permuted RNAs do not resemble the clear cut ends represented in Figure 2. Instead these ends likely have several G residues at the 5′ end and several U residues at the 3′ end. Thus an alternative explanation for the loss of function observed in a subset of the CP mutants is that these positions do not tolerate large additions of nucleotides at these positions.

We purposefully designed the *in vitro* constructs to contain 3 G residues at the 5′ end to ensure efficient T7 polymerase initiation. We took this into consideration in the *Mfold* design of mutants, sometimes adding 3 complementary C residues to prevent predicted misfolding. This information can be found in Supplemental Fig. 1. We also used *Fok*I digestion site to generate a precise 3′ end in all templates for run-off transcription. Because T7 RNA polymerase can add an additional 1 or 2 untemplated nucleotide(s) to the 3′ end of RNA transcripts and there are no more than 3 extra G residues at the 5′ end, the number of additional nucleotides at the novel ends is limited to no more than 5 in total. We also point out that Fig. 6 demonstrates that J1 and J4 can tolerate 5 additional nucleotides in the context of a non-permuted construct *in vitro*. Thus, we feel it is unlikely that the additional nucleotides at the 5′ and 3′ ends are solely responsible for lost functionality of ARC circular permutants.

2) This issue can also extend to the Mini-T results obtained in vivo. I don't know a lot about in vivo RNA processing in yeast, but is it possible that these RNAs do not have well-defined 3′ ends, and that the addition of large RNA sequences in these CP mutants may actually be having a larger effect than the loss of connectivity alone? Certainly the addition of a bulky Sm-binding site at the 3′ end of these constructs could be having very large steric effects beyond simply breaking the connectivity at this position. Nevertheless, it should be noted that the congruency between the in vitro and in vivo data is a good argument in favor of connectivity alone. Still, I think alternative possibilities should be mentioned in the text. The use of possibly just one splint-ligated, chemically synthesized RNA (probably to test connectivity in J4) to test the in vitro results may also be a good idea.

We concur with the reviewer that the striking congruence of results between Micro-T and Mini-T, both *in vitro* and *in vivo*, strongly favors the conclusion that lost function results from loss of RNA connectivity.

As mentioned above, 5 additional nucleotides inserted in J1 or J4, in the context of an non-permuted version of Mini-T(460), did not result in senescence or significantly decreased RNA abundance *in vivo* (Fig. 7A, B). These results indicate that these regions can tolerate insertion of additional nucleotides without adverse effects, suggesting that the sequences introduced at these positions in the relevant cp alleles is not responsible for lost function.

We agree that the circular permutations *in vivo* have heterogeneous 3′ ends, and perhaps even 5′ ends (see above, Referee 1 Major point 2). In fact, our northern blot analysis in Fig. 4B reveals a preponderance of larger (presumably precursor) species, as well as some distinct shorter-than-expected forms. While we feel that it is beyond the scope of this manuscript to map the precise ends of the circularly permuted RNA mutants, it is an interesting future avenue of research that could lead to important insights into the poorly understood process of TLC1 biogenesis and maturation.

We also considered the possibility that repositioning the Sm site to the new 3′ end could cause a "steric hindrance." Specifically, to experimentally test a steric clash of the repositioned Sm site RNA, we linearized the ARC circular permutants with an enzyme that cleaves just upstream of the Sm site, such that the RNA generated would be identical to those shown in Fig. 3B, except lacking the additional (and any) Sm site RNA. In these experiments, we found that the ARC circular permutants still failed to reconstitute detectable telomerase activity *in vitro*. Thus, the additional RNA at the 3′ end is not likely to cause the major defects of these Mini-T circular permutants *in vivo*.

While we would be interested in testing a splint-ligated RNA with precise ends, this is not a technically trivial experiment. In total, we feel our data are sufficient to support our conclusion.

3) My understanding is that while mFold is a useful tool, it is not 100% accurate in determining RNA folding in real-life conditions. I don't think we can completely disregard the possibility that some of these mutations are having an effect on RNA folding in the absence of an alternative method, such as chemical or RNase probing.

We agree with the reviewer that RNA folding predictions such as *Mfold* are certainly not 100% accurate. We directly acknowledged in the Discussion that ARC mutants could be causing RNA misfolding (page 27), and that if there is a role for the ARC in directing proper RNA folding and architecture, this would also be interesting.

It is important to note that structural probing of TLC1 is not routinely done in the field. There are only three existing reports of probing of any portions of TLC1 (Dandjinou *et al*. 2004; Forstemann and Linger, EMBO Rep, 2005; Laterreur *et al.*, NAR, 2013) and none on telomerase generated in rabbit reticulocyte lysates, likely due to associated technical challenges. We have initiated development of chemical and RNase probing assays to investigate the folding of T7 transcribed Micro-T, but these difficult studies, although ongoing, are not near completion.

4) Often times northern blots indicate a substantially reduced RNA accumulation vs. WT mini-T. Can we have an explicit explanation of approximately what levels of Mini-T are required to not show a senescence phenotype? For instance, in Figure 4B, mini-T off of a CEN plasmid shows 15% the levels observed from Mini-T off a 2u plasmid. Were these tested for a senescence phenotype? That would be a strong argument that the 15% observed from a cp-J2 mutant was not the cause for senescence, but rather the specific mutations themselves.

We do show that CEN Mini-T, which is 15% of the 2-micron Mini-T, prevented senescence in Supplemental Fig. 2A. Thus, we feel confident in our conclusion that the low but detectable levels of ARC mutant RNAs should be sufficient to prevent senescence if they were functional. Further, it has been previously published that Mini-T(460) expressed from a CEN plasmid prevents senescence (Zappulla *et al.*, 2005).

Some circularly permuted Mini-T RNAs are less abundant than wild-type Mini-T. We pointed out in the text that levels of 1–10% wild-type TLC1 are sufficient to prevent senescence (e.g., *tlc1-Sm*$^{-}$ at 1–10%, Seto *et al.*, 1999). Based on the quantitation provided in Supplemental Fig. 4, the abundance of all circular permutations are above this level. Additionally, we have based our conclusion on abundance of the mature form of the RNA, which excludes any of the larger or shorter forms of the RNAs, which may or may not be functional. Thus, our quantitation is a conservative estimate of the functional telomerase RNA in the cell.

5) In Figure 4C, many of the mutations that showed a senescence phenotype nevertheless appear to have reasonably long telomeres after 50 generations. I assume the take-home here is that these mutants show telomere shortening at 50 generations that it takes 300 generations to reach in other backgrounds. Perhaps a more informative comparison is between all of the telomerase RNA alleles at 50 generations? In general, perhaps a clearer explanation of what this gel tells us for people in a non-yeast-telomerase background would be useful.

The reviewer is correct in the take-home message for these Southern blots. Senescing mutants are difficult to culture in liquid media prior to growth arrest, but we feel it is always important to show directly that telomeres are shortening at these early time points, to provide evidence that inviability is due to senescence. It is key for readers to note the number of generations that have occurred for each culture of cells, (see also response to Reviewer #3, point 2). To make this clear for the reader, we had underlined the generation time in the Figure, and we now also state this point in the Figure Legend. We hope this helps to clarify this point for a broader readership.

6) In Figure 4A, we see that the cpPKa mutation that was found to be deleterious in vitro has no effect in vivo. Why would this be the case? Is the slight residual activity observed in vitro good enough in vivo?

While cpPKa does have less activity than wild-type Mini-T(460) *in vitro*, it does have reproducibly detectable activity, which we show is sufficient to maintain telomeres *in vivo*. We also point out that the telomeres supported by cpPKa are shorter than those in wild-type Mini-T cells (Fig. 4B, lane 5 v. lane 7), which could be related to decreased activity. However, at least a couple explanations exist to explain why this allele may be more active *in vivo* than *in vitro*. First, it is possible that some fraction of this particular mutant is not folded properly *in vitro*, but that the *in vivo* transcribed RNA is more stably folded. Second, it is possible that in the context of the holoenzyme this particular RNA is more functional (e.g., binding of the essential Est3 protein near the core could promote further function of this mutant *in vivo* that is lacking *in vitro*.

7) I'd be interested to know if there is a sequence-specific requirement in the CEH. It clearly needs to be a helix, as shown in Figure 8. However, does any helix at this region suffice? Or are there specific sequence requirements? This would be useful in determining, for instance, if the CEH is a protein binding site.

We are keen to know the answer to these questions about the core-enclosing helix as well and are currently investigating them, but the work is not yet complete. Once the research is done, we aim to publish it in another paper.

8) A very strong possibility for the defects observed when deleting the 2 nt junction between the CEH and the PK is that the loss of this junction prevents PK folding. With no intervening sequences between the PK and the CEH, it may be that PK folding is being sterically blocked by the CEH. The possibility of the junction influencing RNA folding was covered in the discussion section, however this seems like a very obvious explanation that was not explicitly stated.

We too consider this a possibility and have modified the Discussion accordingly.

9) The discussion mentions the near-universal conservation of core-enclosing helices throughout telomerase RNAs. But in human telomerase RNA, the CEH and the TBE are fused into a single entity, making it difficult to tease apart their relative contributions. Does the human system "count" as an example of the conservation of CEHs?

Indeed, the human telomerase RNA presents an interesting example. Our phylogenetic analysis examined the conservation of the ARC as defined by the data in this manuscript, not just conservation of core-enclosing helices. We define a conserved ARC as (1) having the pseudoknot connected to the template via a core-enclosing helix and template-boundary element and (2) uninterrupted by additional helices. Given the criteria associated with this definition for the ARC, human telomerase qualifies, even though the CEH and TBE are "fused" in this species.

Published data indicate that neither the core-enclosing helix, nor the TBE (i.e., human P1a or P1b), are essential for human telomerase activity (Chen and Greider, 2003). Given this publication, we feel it is possible that the single-stranded RNA between the template and P1b may provide the template boundary definition (as in *T. thermophila*), and that P1b and P1a constitute a single core-enclosing helix element rather than a fused TBE and CEH.

Pre-acceptance letter 16 September 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that both of them consider ther concerns satisfactorily addressed and the manuscript now suitable for publication in The EMBO Journal. The only remaining minor point is referee 3's request to incorporate certain explanation/discussion provided in your response letter also more explicitly into the discussion of the main article itself; please send us a re-revised text file with these additional minor modifications simply via email.
If necessary in light of referee 1's remaining remark (below), you may also wish to send us a modified Supplementary Information file.
Finally, please also complete, sign and send the License to Publish and Page Charge Authorisation forms (see links in my original decision letter), which we require to start the production process.

Once we will have received these files with the last minor changes, we should then be able to swiftly proceed with formal acceptance and publication of the manuscript. I take this opportunity to thank you again for this contribution to The EMBO Journal and congratulate you on a successful publication! Please consider us again in the future for your most exciting work.

Additional correspondence (author) 19 September 2013

I have attached the updated manuscript with text added to the Discussion (p. 24, sentence beginning on line 5) to satisfy Referee 3's remaining request.

Also, we have modified the axis labels to Supplementary Figures 4 and 5 according to Referee 1's helpful suggestion. The updated Supplementary Figures file (i.e., with all Supp. Figs.) is attached.

