EMBO reports

Manuscript EMBO-2015-41708

TERRA promotes telomerase-mediated telomere elongation in Schizosaccharomyces pombe

Martin Moravec, Harry Wischnewski, Amadou Bah, Yan Hu, Na Liu, Lorenzo Lafranchi, Megan King, Claus Azzalin

Corresponding author: Claus Azzalin, ETHZ

Review timeline:

Submission date:	05 November 2015
Editorial Decision:	07 December 2015
Revision received:	19 March 2016
Editorial Decision:	04 April 2016
Revision received:	06 April 2016
Accepted:	07 April 2016

Editor: Esther Schnapp

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 07 December 2015

Thank you for the submission of your manuscript to EMBO reports. We have now received the final referee report, and the full set of reports is copied below.

As you will see, while the referees acknowledge that the addressed questions and some of the findings are interesting, they also point out that the data are insufficient to support the main conclusions and that alternative interpretations cannot be excluded. Recurring concerns are whether polyadenylated TERRA interacting with Trt1 has telomeric repeats, whether the transcription process itself or chromatin modifications may increase telomerase binding to telomeres, and whether telomerase specifically interacts with polyA TERRA. The referees also raise additional, individual concerns and have several suggestions for how the data and conclusions could be strengthened.

From these comments it is clear that publication of the manuscript in our journal cannot be considered at this stage. On the other hand, given the potential interest of your findings, I would like to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Should you decide to embark on such a revision, acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will

otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can publish the study either as a short report or as a full article. For short reports, the revised manuscript text should not exceed 25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view (EV) figures (please note that we changed the name of supplementary data to expanded view). The results and discussion sections must further be combined. For a normal article there are no length limitations, but it should have more than 5 main figures, and the results and discussion sections must be separate.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

REFEREE REPORTS

Referee #1:

In this manuscript, Moravec and colleagues explore the role of telomeric repeat-containing RNA called TERRA in the regulation of telomerase activity, using the fission yeast Schizosaccharomyces pombe as model organism. The authors show that telomere shortening induces expression of TERRA, as previously reported in budding yeast. Interestingly, the authors show that enhanced expression of an engineered telomere promotes its elongation and association with telomerase. The authors propose that a fraction of TERRA can promote telomere elongation through its interaction with telomerase.
The interplay between TERRA expression, telomerase activity and telomere length homeostasis represents an important field of study which still requires to be elucidated. TERRA could represent a missing link in our understanding of telomerase regulation at telomeres. This study thus develops from important and interesting questions. The Azzalin lab has previously used the transcriptionally inducible telomere (tiTEL) assay in mammalian cells to show that induction of TERRA (tiTERRA) expression at a telomere results in elongation of that telomere by telomerase (Farnung et al., PLoS One, 2012). In this manuscript, the authors mostly reproduce these results in S. pombe. The novelty in this study is their observation that tiTERRA expression from a tiTEL induces elongation of the tiTEL by telomerase and correlates with telomerase interaction with tiTERRA. While this work provides more evidence for a role of TERRA in promoting telomerase activity at a specific telomere, it falls short of providing novel mechanistic insights.

Main comments:

- The interaction between TERRA and telomerase is a critical part of this study and the fact that TERRA enrichment in Trt1-myc IP cannot be detected using a telomeric repeat oligo (oC) is intriguing. Results in figure 1 indicate that only a small fraction of TERRA (a polyadenylated fraction) binds telomerase and the authors suggest that, for some reasons, this fraction could contain very short telomeric tract. Does all polyadenylated TERRA contain short telomeric G-tracts in fission yeast? The authors should confirm the presence of a G-rich telomeric repeat tract in Trt1 pull-down, as these repeats is a defining feature of TERRA and discriminates TERRA from ARRET. Did the authors tried to perform Northern blot, or RNA dot blot, on Trt1 IP using telomeric repeat probe? As alternative, they could sequence the polyadenylated RNA bound to Trt1.

- Another concern in this study is the use of two different strains expressing tiTERRA at either one or two telomeres (one or two tiTEL), depending which experiment is performed. Indeed, nearly all Southern blots to quantify telomere elongation are performed with a strain having two tiTELs, while

RT-qPCR and Trt1-myc RIP experiments are performed with a different strain having only one tiTEL. At the end, the authors congregate these data into a coherent model, but they do not provide any data on tiTERRA expression and telomere elongation from a strain containing a unique tiTEL. Also, it is not clear if the strains used for the Telo-PCR and PacBio sequencing experiments have one or two tiTELs. It would be more convincing to include a Southern blot from a strain having only one tiTEL to show the effect of tiTERRA expression on the elongation of that single tiTEL.

- The correlation between tiTERRA overexpression and telomerase-mediated telomere elongation at the engineered telomere is interesting, although the authors cannot rule out the possibility that chromatin modifications or changes in telomere dynamics of the highly transcribed telomere may themselves increase accessibility of telomerase to the telomere. The authors should discuss these possibilities in the text.

- In budding yeast, similar experiments have been performed using either a GAL-inducible promoter or a Tet-responsive promoter inserted upstream of a single telomere (Sandell et al., PNAS, 1994, Pfeiffer and Lingner, PLoS Genet, 2012, Maicher at al., NAR, 2012). In these articles, telomere shortening was observed when transcription was induced. Why is it different in fission yeast? The authors should comment on these observations.

Minor comments:

- Pages 5 and 6 : results from Trt1-myc RIP experiments refer to figure 1b (not figure 2b).

- Page 6, line 3 : "Our inability to detect a statistically significant accumulation of total TERRA in Trt1-myc RIP samples could arise from the fact that polyadenylated TERRA constitutes only a minor fraction of total TERRA". Do you mean non-polyadenylated TERRA instead?

- Page 12, figure 4b: "Similarly to endogenous TERRA (Fig 1b), polyadenylated tiTERRA preferentially associated with telomerase in cells (Fig. 4b). " Where do you see that polyA+ tiTERRA associates preferentially with Trt1 in this figure? I don't see much difference between total tiTERRA and polyA+ tiTERRA in the Trt1-myc RIP.

Referee #2:

Telomeres are transcribed into non-coding RNA species, dubbed TERRA, that play multiple roles at telomeres. Conflicting data have been reported regarding the putative role of TERRA in regulating either telomerase recruitment at telomeres or telomerase activity. The study by Moravec et al aims at investigating the impact of telomere transcription on telomerase recruitment at telomeres of fission yeast cells. The main results of the study are that 1) fission yeast telomerase binds polyA+ TERRA species, 2) transcription of telomeres into polyA+ TERRA increases in telomerase-deficient cells experiencing telomere shortening, 3) forced transcription of one given telomere through insertion of an inducible nmt1 promoter in combination with TSA treatment leads to specific elongation of that telomere in a telomerase-dependent manner. Based on these results, authors propose that interaction of telomerase with polyA+ TERRA promotes telomerase recruitment to shortening telomeres.

Overall, the study is interesting and provides some nice data. I, however, have a series of major concerns that I list below and that should be taken into account by authors to hopefully improve the study and strengthen the conclusions.
1) Why is telomerase specifically interacting with polyA+ TERRA? Authors propose that interaction of trt1 (fission yeast catalytic subunit of telomerase) with polyA+ TERRA may occur in the nucleoplasm and provide a cargo for trt1 on its way to telomeres. This view is actually displayed in the cartoon model of Fig 4d. In that scenario, one may assume that nucleoplasmic TERRA may be the only species available to interact with trt1. However, to my knowledge, there is no proof that polyA+ TERRA is in the nucleoplasm of fission yeast cells. This should be investigated by subcellular fractionation experiments. More importantly, Fig 1b shows that, in RIP experiments, polyA+ TERRA species are pulled-down with myc-tagged telomerase but, on the other hand, using the same primers for qPCR (oF1+oR1), no TERRA could be amplified when RT was performed with telomeric primers instead of oligodT primers. This does not make sense to me. Authors argue that this observation may be explained by the fact that polyA+ TERRA only constitute a minor

fraction of total TERRA but the argument is flawed in this case as qRT-PCR data were not normalized to input RNA but to RNA recovered in pull down experiments performed in untagged strains. In other words, if canonical polyA+ TERRA species (by canonical, I mean species including both subtelomeric sequences AND telomeric repeats) were indeed associated with trt1, and if polyA+ TERRA were the only species able to interact with trt1, qRT-PCR should provide similar results whether TERRA species are polyadenylated or not. One possible explanation would therefore be that trt1 interacts with sub-TERRA species that do NOT contain telomeric repeats, a kind of ARRET molecules that would start at TERRA TSS and stop before telomeric repeats. This hypothesis should be tested by dot blotting RIP samples and probing with a telomeric probe.
2) In tiTEL cells with forced transcription of telomeres, the experimental settings are such that a strong promoter is introduced near the telomere and this may possibly impact on telomerase recruitment through chromatin remodeling of subtelomeres. Hence, the transcription process per se may possibly help telomerase recruitment. In line with the above comment, it is also possible that, in this experimental setting, thiamine deprivation-induced transcription of polyA+ sub-TERRA species (with no telomeric repeats) may promote telomerase recruitment. To address these hypotheses, it would be nice to insert transcription termination signals either directly downstream of Pnmt1 promoter sequence or directly upstream of telomeric repeats (see Fig 2a). It should be noted that, in tiTEL cells, TERRA interaction with myc-tagged trt1 was only convincing when RT was performed with oligodT primers (in the absence of TSA treatment which, I believe, are closer to physiological settings).
3) Figure 3 suggests that tiTEL telomere is specifically elongated in induced conditions and this, in a trt1-dependent manner. Accordingly, Fig 4 shows that trt1 is recruited at tiTEL when transcription is induced. I feel however that it is important to quantify non-tiTEL DNA in trt1-myc ChIP experiments to further validate these observations. This should be done with primers that amplify other subtelomeric loci of fission yeast.
4) It would have been interesting to perform RIP experiments in either trt1-myc ter1D or trt1-myc est1D cells to see whether, under these more physiological conditions of telomere transcription up-regulation, TERRA binding to trt1 is indeed increased.
5) A report from the Azzalin's group published in 2012 (Bah et al, NAR) identified Cid12 and Cid14 non-canonical poly(A) polymerases as regulators of fission yeast TERRA. Did the authors test whether Cid deletion had any impact on polyA+ TERRA abundance, trt1 interaction and telomere length?

Minor comments:
-Page 5 and page 6: Fig 1b instead of Fig 2b
-Page 6 specifically functionally
-Fig 2b: I do not understand the difference in nTEL signals between WT and tiTEL cells: are cell lines thus really comparable?
-Has RNA spike been added in RIP experiments to control for RNA extraction and cDNA synthesis efficiency?
-Fig 3c: Why not showing data for tiTEL trt1D cells in +/-THI+/-TSA?

Referee #3:

The study by Moravec and colleagues attempts to elucidate the role of the non-coding telomeric TERRA transcripts in telomere length maintenance in Schizosaccharomyces pombe. The authors show that TERRA associates with Trt1 in RNA immunoprecipitation experiments and that TERRA transcription increases at short telomeres. They further generated a transcriptionally inducible telomere by introducing the nmt1+ promoter upstream of the TERRA transcription site. They demonstrate that increased expression of TERRA leads to elongation of telomeres. From these data, the authors proposed a model whereby shortening of telomeres and consecutive loss of Rap1 repression would increase TERRA transcription and nucleoplasm release of poly-adenylated TERRA. The latter would interact with telomerase and bring it back to its telomere of origin favoring telomere elongation. This model also relies on previous work carried out both in yeast and human, especially the work from Cusanelli et al. (Mol Cell, 2013), where they propose that TERRA nucleates telomerase molecules into clusters prior to their recruitment at a short telomere.
Such model of telomerase recruitment facilitated by TERRA is quite exciting. However, at this stage of the manuscript this model remains too speculative. Addressing the following issues should strengthen it.

1) One major conclusion of the work is that TERRA stimulates telomerase recruitment and activity at chromosome ends. An alternative model would be that TERRA induced transcription specifically delocalizes Shelterin components from the transcribed telomere resulting in the elongation of this telomere by relieving the inhibition exerted by the Shelterin. The authors should analyse the binding of Taz1 (or Rap1, or Poz1) by ChIP and show that the transcribed telomere has similar level of Taz1 than the control telomeres provided that specific primers can be found to distinguish the transcribed telomere from the control telomeres.

2) The authors convincingly show by RIP that TERRA interacts with Trt1. It is less clear from the results of the MS that nucleoplasm released TERRA transcripts interact with telomerase. Could it be possible that telomeric DNA mediates the interaction between TERRA and Trt1? This could be tested by DNase treatment of the extract before immunoprecipitation.

3) It has been shown that TERRA can form RNA/DNA hybrids at telomeres. Another possibility would be that TERRA transcription per se or the presence of TERRA/DNA hybrids might stabilize an open state at short telomeres and hence the recruitment of telomerase. This alternative model could be tested by by testing whether telomere elongation upon tiTERRA transcription is abolished by RNaseH overexpression.

4) It is not clear why TERRA transcripts can be detected in the Trt1 immunoprecipitates only when using a poly-T for reverse transcription and not with the telomeric specific primer oC. A similar discrepancy is observed at the inducible telomere (Fig 4). Accordingly the authors measured a 6 fold increase of total tiTERRA transcription and a 50 fold increase in poly(A)+ tiTERRA upon induction of the nmt1+ promoter. The size of the tiTERRA transcripts (300-400 bases) measured by northern blot (Fig 2e) is also surprising considering the 360 bases (of subtelomeric sequence) that the transcripts are supposed to contain according to the Fig2c. Together these observations suggest that the detected transcripts contain very few telomeric repeats. This point should be further discussed in the manuscript. Do the author know whether Trt1 interacts with TERRA independently of the presence of Ter?

5) The demonstration that only TiTEL is elongated upon TERRA transcription is not completely clear. The authors mention in the text that the length of "the pure telomeric repeats" within TiTELs (as the nTELs) is around 200bp. However, by referring to fig S1b and S1c, the average length of TiTEL is about 300bp. Could the authors explain this discrepancy? Also in Fig S1c lane5, telomeres in the titel strain look more heterogenous (with shorter telomeres) compared to WT strain (compare lane 2 to lane 5). From the figures, it is not clear whether the TiTELs have the same telomere length than the nTELs. If TiTELs are shorter, it may that they will be preferentially elongated. This point needs to be clarified.

6) Authors have deleted the trt1 to show that elongation of telomeres after TSA treatment and induction is telomerase-dependent. A slight elongation of telomere is still observed in presence of TSA. This elongation is not rad51 or exo1-dependant. Their conclusion is that TSA may provoke telomerase-independent elongation. Another possibility would be that senescence is delayed in presence of TSA thereby explaining the difference observed in telomere length in trt1 deleted strain. It would be helpful to understand how experience has been performed and to indicate at which stage of the senescence samples have been collected. It would have been preferable to use the tpz1K75A mutant strain that display short but stable telomeres.

Other points:
1) Figure 1C: It is surprising to observe that telomeric fragments run at an average of 400bp after Telo PCR. WT telomeric repeats run at approximately 300bp (as shown by Apa1 telobot Figure S1c lane 2).
2) The authors do not mention how do they perform senescence (liquid vs agar plate), and how they follow population doublings. Senescence curves might be informative.
3) In general, the MS is rather difficult to read. The figure legends need to be more detailed (for example Figure1). Also in Figure 3, lane numbers should be added to the figure and referred in the text.

1st Revision - authors' response 19 March 2016

Thank you very much for giving us the opportunity to submit a revised version of our manuscript entitled 'TERRA promotes telomerase-mediated telomere elongation in *Schizosaccharomyces pombe*' [Paper #EMBOR-2015-41708V1]. The Reviewers' comments were extremely constructive, prompting us to dig deeper into the intriguing question of how TERRA impacts telomerase activity at telomeres, for which a unified model is still lacking. We have now addressed the vast majority of the Reviewers' comments through the inclusion of additional data (see point-by-point response below). Gratifyingly, we believe that these additions have very much improved the manuscript, thereby clarifying and deepening the manner in which this work extends our understanding of how telomeric transcription and TERRA support telomerase-mediated telomere elongation.

To accommodate the Reviewers' requests, the manuscript is now substantially longer and contains 5 main Figures and 4 Expanded View Figures. Our revised manuscript comprises approximately 4400 characters (including spaces but excluding references and materials and methods) and we would therefore prefer to publish it as a full article.

Point-by-point response to the Reviewers' comments

Referee #1:
In this manuscript, Moravec and colleagues explore the role of telomeric repeat-containing RNA called TERRA in the regulation of telomerase activity, using the fission yeast Schizosaccharomyces pombe as model organism. The authors show that telomere shortening induces expression of TERRA, as previously reported in budding yeast. Interestingly, the authors show that enhanced expression of an engineered telomere promotes its elongation and association with telomerase. The authors propose that a fraction of TERRA can promote telomere elongation through its interaction with telomerase. The interplay between TERRA expression, telomerase activity and telomere length homeostasis represents an important field of study which still requires to be elucidated. TERRA could represent a missing link in our understanding of telomerase regulation at telomeres. This study thus develops from important and interesting questions. The Azzalin lab has previously used the transcriptionally inducible telomere (tiTEL) assay in mammalian cells to show that induction of TERRA (tiTERRA) expression at a telomere results in elongation of that telomere by telomerase (Farnung et al., PLoS One, 2012). In this manuscript, the authors mostly reproduce these results in S. pombe. The novelty in this study is their observation that tiTERRA expression from a tiTEL induces elongation of the tiTEL by telomerase and correlates with telomerase interaction with tiTERRA. While this work provides more evidence for a role of TERRA in promoting telomerase activity at a specific telomere, it falls short of providing novel mechanistic insights.

We are very happy that this Reviewer recognizes the importance of the subject and our contribution to this ongoing area of research. We are also convinced that TERRA is a missing link in telomerase biology, and the revised version of our manuscript, in which the comments of the Reviewers have been addressed, should help deepen our understanding in this important area. However, we respectfully disagree with the statement that we are mostly reproducing the results of our PLoS One paper (Farnung et al. 2012), as here we are presenting the first direct evidence that TERRA and/or telomere transcription stimulates telomerase-mediated elongation. While in our PLoS One paper we showed that increasing transcription of a tiTEL seeded in HeLa cells did not prevent telomerase from elongating it, we did not detect a difference in length between induced and uninduced tiTELs. Given the observations in fission yeast presented in this manuscript, we now think this result is likely attributable to the fact that telomeres in the chosen HeLa cells were too long to allow detection of subtle changes in length. This point is now clarified in the introduction (page 4).

Main comments:
- The interaction between TERRA and telomerase is a critical part of this study and the fact that TERRA enrichment in Trt1-myc IP cannot be detected using a telomeric repeat oligo (oC) is intriguing. Results in figure 1 indicate that only a small fraction of TERRA (a polyadenylated fraction) binds telomerase and the authors suggest that, for some reasons, this fraction could contain very short telomeric tract. Does all polyadenylated TERRA contain short telomeric G-tracts in fission yeast? The authors should confirm the presence of a G-rich telomeric repeat tract in Trt1 pull-down, as these repeats is a defining feature of TERRA and discriminates TERRA from ARRET.

Did the authors tried to perform Northern blot, or RNA dot blot, on Trt1 IP using telomeric repeat probe? As alternative, they could sequence the polyadenylated RNA bound to Trt1.

This is indeed a very critical point and we would like to thank all Reviewers for pointing it out and inciting us to follow this direction. We have performed 3' end RACE of total poly(A)+ RNA or poly(A)+ RNA from Trt1-myc immunoprecipitated fractions, both in wt and *ter1*D cells. Unfortunately, despite sequencing more than 150 RACE clones for each condition, we were unable to identify TERRA in samples corresponding to wt total RNA and to both IP fractions, most likely due to the extremely low abundance of polyA+ TERRA in these samples. In samples from *ter1*D total poly(A)+ RNA, where polyA+ TERRA is 20-to-50 fold more abundant, we identified several TERRA sequences. Surprisingly, but consistent with the data presented in the initial manuscript, we discovered that polyA+ TERRA is largely devoid of telomeric repeats. These data explain why we could not detect TERRA in trt1-myc pull-downs when qPCRs were performed on cDNA reverse transcribed with C-rich telomeric oligonucleotides (oC). Although we were unable to sequence polyA+ TERRA 3' ends in wt cells and in Trt1-myc immunoprecipitated fractions, our RIP/qRT-PCR data using oC or odT oligonucleotides indicate that: *i*) it is specifically the polyA+ TERRA molecules largely devoid of telomeric repeats that preferentially interact with telomerase and *ii*) this is the case not only in *ter1*D but also in wt cells. These additional data are presented in Figure 1F and in the Results section (pages 6-8), and discussed throughout the Discussion section. Moreover, for sake of accuracy and clarity, we now refer to TERRA containing telomeric repeat tracts long enough to allow priming with oC oligonucleotides as 'G-rich TERRA' rather than 'total TERRA'.

- Another concern in this study is the use of two different strains expressing tiTERRA at either one or two telomeres (one or two tiTEL), depending which experiment is performed. Indeed, nearly all Southern blots to quantify telomere elongation are performed with a strain having two tiTELs, while RT-qPCR and Trt1-myc RIP experiments are performed with a different strain having only one tiTEL. At the end, the authors congregate these data into a coherent model, but they do not provide any data on tiTERRA expression and telomere elongation from a strain containing a unique tiTEL. Also, it is not clear if the strains used for the Telo-PCR and PacBio sequencing experiments have one or two tiTELs. It would be more convincing to include a Southern blot from a strain having only one tiTEL to show the effect of tiTERRA expression on the elongation of that single tiTEL.

The original tiTEL strain integrated *nmt1* promoters at two subtelomeres (right arm of chromosome I and left arm of chromosome II; see Figure EV1A). This is due to the fact that 4 out of the 6 *pombe* subtelomeres are identical in sequence, while the sequence of the remaining two telomeres of chromosome III are unknown because of the presence of long stretches of rDNA repeats. The clones generated with single tiTEL insertions were isolated by backcrossing of the 2 tiTEL strain to wt. In general, we would argue that the use of different strains (with 1 or 2 tiTELs) provides added value, as it shows that telomere transcription affects telomere length in a strain-independent manner. Still, we appreciate the comment of the Reviewer. In the revised manuscript we have both clarified the strains and the number of tiTELs used in each experiment in the corresponding figure legend, while also providing further experimental comparisons of the 1 and 2 tiTEL strains. For example, we have now added Telo-PCR data showing that tiTEL lengthening occurs with similar dynamics in untagged strains with 1 and 2 tiTELs (EV1E) as well as in the 1 tiTEL strain utilized for Trt1-myc RIPs (Figure 5B). We have also added qRT-PCR data for the original strain carrying 2 tiTELs (Figure 3D), which was utilized for Southern blot analysis of tiTELs (Figure 4A and B) and northern blot analysis of tiTERRA (Figure 3E). Moreover, all strains used in Figure EV2 carry 1 tiTEL, further confirming that tiTEL elongation is not clone specific.

- The correlation between tiTERRA overexpression and telomerase-mediated telomere elongation at the engineered telomere is interesting, although the authors cannot rule out the possibility that chromatin modifications or changes in telomere dynamics of the highly transcribed telomere may themselves increase accessibility of telomerase to the telomere. The authors should discuss these possibilities in the text.

This is also a very good point. We have built our working model of TERRA-mediated telomerase recruitment not only on the data presented in this study, but also on previous data from my laboratory as well as on reports from other groups. Taken these studies together, we suggest the possibility that TERRA binding to telomerase is involved in telomerase recruitment to telomeres. Nevertheless, we do not intend to rule out the equally plausible, attractive (and not mutually

exclusive) possibility that transcription per se could also contribute to increase the accessibility of the telomere to telomerase, for example by diminishing the density of telomere-bound telomerase inhibitors, by altering the landscape of telomeric chromatin modifications, or by promoting telomere dynamics within the nucleus. We now discuss this possibility in the Discussion (page 14).

- In budding yeast, similar experiments have been performed using either a GAL-inducible promoter or a Tet-responsive promoter inserted upstream of a single telomere (Sandell et al., PNAS, 1994, Pfeiffer and Lingner, PLoS Genet, 2012, Maicher at al., NAR, 2012). In these articles, telomere shortening was observed when transcription was induced. Why is it different in fission yeast? The authors should comment on these observations.

This is true and the point was not sufficiently discussed in the original version of the manuscript. We speculate that Exo1 might be the key to explain this discrepancy. Indeed, while the Luke and Lingner laboratories showed that in budding yeast tiTEL transcription causes telomere shortening in a telomerase-independent and Exo1-dependent manner, we find no evidence that Exo1 affects tiTEL dynamics in fission yeast (EV2). Thus TERRA and/or telomere transcription in independent species might have acquired different functions in modulating activities at chromosome ends (see Discussion, page 16).

Minor comments:
- Pages 5 and 6 : results from Trt1-myc RIP experiments refer to figure 1b (not figure 2b).

This has been corrected.

- Page 6, line 3 : "Our inability to detect a statistically significant accumulation of total TERRA in Trt1-myc RIP samples could arise from the fact that polyadenylated TERRA constitutes only a minor fraction of total TERRA". Do you mean non-polyadenylated TERRA instead?

This speculation has been removed, as we now show that polyA+ TERRA is largely devoid of telomeric repeat sequences.

- Page 12, figure 4b: "Similarly to endogenous TERRA (Fig 1b), polyadenylated tiTERRA preferentially associated with telomerase in cells (Fig. 4b). " Where do you see that polyA+ tiTERRA associates preferentially with Trt1 in this figure? I don't see much difference between total tiTERRA and polyA+ tiTERRA in the Trt1-myc RIP.

Indeed, polyA+ tiTERRA preferentially associates with Trt1-myc in THI- TSA- samples, but not in THI- TSA+ samples. This suggests that forcing RNAPII transcription (THI withdrawal) in the presence of TSA might lead to production of polyA+ tiTERRA containing telomeric stretches long enough to permit oC reverse transcription. This interpretation is consistent with the corresponding tiTERRA qRT-PCR data (Figure 5A). We now clarify and further discuss this point in the text (Results, page 13).

Referee #2:
Telomeres are transcribed into non-coding RNA species, dubbed TERRA, that play multiple roles at telomeres. Conflicting data have been reported regarding the putative role of TERRA in regulating either telomerase recruitment at telomeres or telomerase activity. The study by Moravec et al aims at investigating the impact of telomere transcription on telomerase recruitment at telomeres of fission yeast cells. The main results of the study are that 1) fission yeast telomerase binds polyA+ TERRA species, 2) transcription of telomeres into polyA+ TERRA increases in telomerase-deficient cells experiencing telomere shortening, 3) forced transcription of one given telomere through insertion of an inducible nmt1 promoter in combination with TSA treatment leads to specific elongation of that telomere in a telomerase-dependent manner. Based on these results, authors propose that interaction of telomerase with polyA+ TERRA promotes telomerase recruitment to shortening telomeres. Overall, the study is interesting and provides some nice data. I, however, have a series of major concerns that I list below and that should be taken into account by authors to hopefully improve the study and strengthen the conclusions.

We are very happy that this Reviewer finds our study interesting and we would like to thank her/him

for the suggestions. We believe that our manuscript is now much stronger and more insightful than the initial submission.

1) Why is telomerase specifically interacting with polyA+ TERRA? Authors propose that interaction of trt1 (fission yeast catalytic subunit of telomerase) with polyA+ TERRA may occur in the nucleoplasm and provide a cargo for trt1 on its way to telomeres. This view is actually displayed in the cartoon model of Fig 4d. In that scenario, one may assume that nucleoplasmic TERRA may be the only species available to interact with trt1. However, to my knowledge, there is no proof that polyA+ TERRA is in the nucleoplasm of fission yeast cells. This should be investigated by subcellular fractionation experiments.

Indeed, our model was based on data from the Lingner laboratory showing that polyA+ TERRA accumulates in the nucleoplasm of human cancer cells. We have now performed experiments where we have fractionated fission yeast cellular material into soluble and insoluble components. As expected, chromatin was found to be strongly enriched in insoluble fractions. qRT-PCR anaylsis of RNA extracted from the different fractions revealed that polyA+ TERRA is predominantly soluble, and thus able to freely diffuse throughout the nucleoplasm. These new data are presented in Figure 1G and H and in the Results section (page 7).

More importantly, Fig 1b shows that, in RIP experiments, polyA+ TERRA species are pulled-down with myc-tagged telomerase but, on the other hand, using the same primers for qPCR (oF1+oR1), no TERRA could be amplified when RT was performed with telomeric primers instead of oligodT primers. This does not make sense to me. Authors argue that this observation may be explained by the fact that polyA+ TERRA only constitute a minor fraction of total TERRA but the argument is flawed in this case as qRT-PCR data were not normalized to input RNA but to RNA recovered in pull down experiments performed in untagged strains. In other words, if canonical polyA+ TERRA species (by canonical, I mean species including both subtelomeric sequences AND telomeric repeats) were indeed associated with trt1, and if polyA+ TERRA were the only species able to interact with trt1, qRT-PCR should provide similar results whether TERRA species are polyadenylated or not. One possible explanation would therefore be that trt1 interacts with sub-TERRA species that do NOT contain telomeric repeats, a kind of ARRET molecules that would start at TERRA TSS and stop before telomeric repeats. This hypothesis should be tested by dot blotting RIP samples and probing with a telomeric probe.

We would like to thank all Reviewers for pointing out this critical issue and inciting us to follow this direction. Please see our response to 'Main comment 1' of Reviewer 1, which addresses this important point.

2) In tiTEL cells with forced transcription of telomeres, the experimental settings are such that a strong promoter is introduced near the telomere and this may possibly impact on telomerase recruitment through chromatin remodeling of subtelomeres. Hence, the transcription process per se may possibly help telomerase recruitment. In line with the above comment, it is also possible that, in this experimental setting, thiamine deprivation-induced transcription of polyA+ sub-TERRA species (with no telomeric repeats) may promote telomerase recruitment. To address these hypotheses, it would be nice to insert transcription termination signals either directly downstream of Pnmt1 promoter sequence or directly upstream of telomeric repeats (see Fig 2a). It should be noted that, in tiTEL cells, TERRA interaction with myc-tagged trt1 was only convincing when RT was performed with oligodT primers (in the absence of TSA treatment which, I believe, are closer to physiological settings).

These are very interesting suggestions and indeed we have previously attempted to design such experiments. However, our experience with fission yeast and cultured human cells has taught us that generating cell clones where transcription of TERRA is suppressed through introduction of terminator sequences or by eliminating promoter activity is highly perturbing, as we consistently observe that such clones have abnormal telomeres that prevent valuable experimental comparisons. Indeed it was these observations that prompted us to opt for an inducible system where TERRA transcription can be experimentally manipulated within the same cells, thus allowing direct comparison of cells only differing in TERRA transcription levels. Moreover, as addressed in the 'Main comment 3' of Reviewer 1, we now clearly acknowledge that transcription per se could also contribute to increase telomerase accessibility (Discussion, page 14).

3) Figure 3 suggests that tiTEL telomere is specifically elongated in induced conditions and this, in a trt1-dependent manner. Accordingly, Fig 4 shows that trt1 is recruited at tiTEL when transcription is induced. I feel however that it is important to quantify non-tiTEL DNA in trt1-myc ChIP experiments to further validate these observations. This should be done with primers that amplify other subtelomeric loci of fission yeast.

Although we agree with this point in principle, it is unfortunately not possible to perform the suggested analysis. The sequences of all *pombe* subtelomeres are identical (except for possibly the ones of chromosome III, which are not fully sequenced due to the presence of long stretches of rDNA). To remain as close as possible to the wt situation, we decided to insert the inducible *nmt1* promoter in a way that would only minimally alter the subtelomeric sequence residing between TERRA/tiTERRA TSS and the telomeric tract (see Figure 3A and C). Thus, while we can specifically amplify tiTEL sequences, we cannot design oligonucleotides that amplify only natural subtelomeres and not tiTELs, unless we use oligonucleotide pairs that amplify very long fragments of approximately 1 kb (this is shown in Figure EV3). Our ChIP protocol includes a crucial sonication step that shears DNA below 500 bp, thus precluding the use of such oligonucleotide pairs in this context. Nevertheless, we believe that we have very clearly shown that tiTERRA transcription induces telomere lengthening only *in cis* (Figure 4A and B, Figure EV1D and Figure EV3D).

4) It would have been interesting to perform RIP experiments in either trt1-myc ter1D or trt1-myc est1D cells to see whether, under these more physiological conditions of telomere transcription up-regulation, TERRA binding to trt1 is indeed increased.

We have now performed RIP experiments in *ter1*D cells and observe a decrease in the fraction of polyA+ TERRA associated with Trt1-myc when expressed relative to the input. However, this fractional decrease is misleading, as it is a consequence of the higher amount of total polyA+ TERRA in *ter1*D mutant cells. Normalizing instead to the stable ACT1 mRNA in the corresponding inputs reversed this effect. These data are presented in Figure 2C and reveal that TER1 is dispensable for the TERRA/Trt1-myc interaction. Moreover, increasing the cellular pool of polyA+ TERRA is not sufficient to increase TERRA/Trt1-myc complexes. This suggests that telomerase levels may be limiting in cells and that TERRA and/or telomere transcription may allow 'funneling' of the few molecules of active telomerase within a cell specifically to the shortest telomeres (Results, page 8).

5) A report from the Azzalin's group published in 2012 (Bah et al, NAR) identified Cid12 and Cid14 non-canonical poly(A) polymerases as regulators of fission yeast TERRA. Did the authors test whether Cid deletion had any impact on polyA+ TERRA abundance, trt1 interaction and telomere length?

We have analyzed TERRA levels and telomere length in *cid12+* and *cid14+* deleted strains (see Figure R1). Deletion of *cid12+* increased G-rich TERRA (~ 7 fold increase) but not polyA+ TERRA while deletion of *cid14+* increased both G-rich (~ 93 fold) and polyA+ TERRA (~ 9.5 fold). Telomere length was not evidently altered in *cid12*D cells while telomeres were slightly shorter in *cid14*D than in wt cells. These results confirm that both non canonical poly(A) polymerases counteract TERRA accumulation in cells. Nevertheless a straightforward correlation between polyA+ TERRA levels and telomere length is not evident. Although we find these results very intriguing, we would prefer not to include them in our manuscript, as they seem too preliminary and not very helpful at this stage. They will constitute the foundation for future experiments aiming at identifying the RNA cleavage and polyadenylation machineries involved in polyA+ TERRA production when telomeres shorten.

[Data removed upon author's request]

Minor comments:
-Page 5 and page 6: Fig 1b instead of Fig 2b

This has been corrected.

-Page 6 specifically functionally

This has been changed.

-Fig 2b: I do not understand the difference in nTEL signals between WT and tiTEL cells: are cell lines thus really comparable?

These signals correspond to nTELs carrying a polymorphic *Hind*III site not present on the other nTELs, which are therefore retained in the upper part of the gel when DNA is digested with *Hind*III. The presence of polymorphic *Hind*III sites is not uncommon at fission yeast telomeres and in our case allows simultaneous visualization of tiTELs and nTELs of the same cells as two populations of distinct yet comparable sizes. Moreover, comparisons between wt and tiTEL strains are mostly used to show that the latter indeed contain tiTELs while the main conclusions are dragged from comparing isogenic tiTEL strains treated or not with thiamine and TSA. To directly compare how nTELs from tiTEL and wt cells respond to thiamine and TSA we performed Southern blot hybridization of genomic DNA digested with ApaI, thus releasing all nTELs at the bottom of the gel in both strains (Fig EV1D).

-Has RNA spike been added in RIP experiments to control for RNA extraction and cDNA synthesis efficiency?

No, spiking RNA in extracts for RIPs is not included in our protocol. Nevertheless, detection of TER1 in Trt1-myc pull-downs serves as an important control to demonstrate the efficiency and reproducibility of our protocol (Figure 1A and Figure EV4A). Similarly, the lack of detectable ACT1 mRNA in the same material confirms the specificity of our RIPs (Figure 1A and Figure EV4A).

-Fig 3c: Why not showing data for tiTEL trt1D cells in +/-THI+/-TSA?

We did not perform PacBio sequencing of tiTELs from *trt1*D cells grown in presence of TSA because the differences in telomere length induced by TSA alone and by combining TSA with thiamine are already very evident in our Southern blots (Figure 3B).

Referee #3:
The study by Moravec and colleagues attempts to elucidate the role of the non-coding telomeric TERRA transcripts in telomere length maintenance in Schizosaccharomyces pombe. The authors show that TERRA associates with Trt1 in RNA immunoprecipitation experiments and that TERRA transcription increases at short telomeres. They further generated a transcriptionally inducible telomere by introducing the nmt1+ promoter upstream of the TERRA transcription site. They demonstrate that increased expression of TERRA leads to elongation of telomeres. From these data, the authors proposed a model whereby shortening of telomeres and consecutive loss of Rap1 repression would increase TERRA transcription and nucleoplasm release of poly-adenylated TERRA. The latter would interact with telomerase and bring it back to its telomere of origin favoring telomere elongation. This model also relies on previous work carried out both in yeast and human, especially the work from Cusanelli et al. (Mol Cell, 2013), where they propose that TERRA nucleates telomerase molecules into clusters prior to their recruitment at a short telomere. Such model of telomerase recruitment facilitated by TERRA is quite exciting. However, at this stage of the manuscript this model remains too speculative. Addressing the following issues should strengthen it.

We are very happy that this Reviewer recognizes the value of our study and we appreciate her/his insightful suggestions.

1) One major conclusion of the work is that TERRA stimulates telomerase recruitment and activity at chromosome ends. An alternative model would be that TERRA induced transcription specifically delocalizes Shelterin components from the transcribed telomere resulting in the elongation of this telomere by relieving the inhibition exerted by the Shelterin. The authors should analyse the binding of Taz1 (or Rap1, or Poz1) by ChIP and show that the transcribed telomere has similar level of Taz1 than the control telomeres provided that specific primers can be found to distinguish the transcribed

telomere from the control telomeres.

We have now performed ChIPs in tiTEL strains expressing GFP-tagged Taz1 and found that Taz1 binding to tiTELs was slightly diminished, although not in a statistically significant manner, when cells were grown in presence of TSA while it was completely unaffected by THI levels (Figure EV4C and D; Results, page 13). It is therefore unlikely that tiTERRA-mediated tiTEL elongation is a consequence of diminished binding of Taz1 to tiTELs. As for comparing binding of Taz1-GFP to tiTELs and nTELs, unfortunately this analysis is not possible, as we cannot design PCR oligonucleotides allowing distinction between tiTELs and nTELs using DNA extracted from sonicated chromatin (see also our response to Reviewer 2's point 3). Nevertheless, it seems clear that transcription-mediated tiTEL elongation cannot be ascribed to Taz1 displacement from the same tiTELs when thiamine is withdrawn. Moreover, in the current version of the Discussion we also acknowledge the non-mutually exclusive possibility that TERRA transcription per se could also increase the accessibility of the telomere to telomerase, for example by diminishing the density of telomere-bound telomerase inhibitors (page 14).

2) The authors convincingly show by RIP that TERRA interacts with Trt1. It is less clear from the results of the MS that nucleoplasm released TERRA transcripts interact with telomerase. Could it be possible that telomeric DNA mediates the interaction between TERRA and Trt1? This could be tested by DNase treatment of the extract before immunoprecipitation.

We have now performed RIP experiments where immunocomplexes bound to beads were extensively treated with DNaseI prior to RNA extraction. DNaseI treatments did not affect the yields of polyA+ TERRA co-immunoprecipitated with Trt1-myc (Figure 2B; Results, page 7), strongly implying that DNA does not bridge TERRA to Trt1-myc. Moreover, we now show that polyA+ TERRA is enriched in soluble cellular fractions (Figure 1G and H; see also our response to Reviewer 2's point 1), further supporting the hypothesis that interactions between polyA+ TERRA and telomerase are established in the nucleoplasm.

3) It has been shown that TERRA can form RNA/DNA hybrids at telomeres. Another possibility would be that TERRA transcription per se or the presence of TERRA/DNA hybrids might stabilize an open state at short telomeres and hence the recruitment of telomerase. This alternative model could be tested by testing whether telomere elongation upon tiTERRA transcription is abolished by RNaseH overexpression.

This is of course a very interesting point and we are currently working on these lines of research both in fission yeast and human cells. However, it is unlikely that RNA:DNA hybrids positively regulate telomerase activity at telomeres. The Lingner laboratory has previously shown in budding yeast that suppressing the RNA exonuclease Rat1 causes telomere shortening through telomerase inhibition and that over-expression of the RNaseH enzyme Rnh201p in the same cells averts telomerase inhibition (Luke et al. *Mol Cell* 32, 2008). We also know that over-expression of Rnh201 in fission yeast cells induces progressive elongation of telomeres (Figure R2). Overall it seems that RNA:DNA hybrids restrict telomerase activity at telomeres, rather then promoting it. We are very intrigued by these data and, as mentioned, we are following them up. Nevertheless, at this stage, they are too preliminary and would not add to the current study.

[Data removed upon author's request]

4) It is not clear why TERRA transcripts can be detected in the Trt1 immunoprecipitates only when using a poly-T for reverse transcription and not with the telomeric specific primer oC. A similar discrepancy is observed at the inducible telomere (Fig 4). Accordingly the authors measured a 6 fold increase of total tiTERRA transcription and a 50 fold increase in poly(A)+ tiTERRA upon induction of the nmt1+ promoter. The size of the tiTERRA transcripts (300-400 bases) measured by northern blot (Fig 2e) is also surprising considering the 360 bases (of subtelomeric sequence) that the transcripts are supposed to contain according to the Fig2c. Together these observations suggest that the detected transcripts contain very few telomeric repeats. This point should be further discussed in the manuscript. Do the author know whether Trt1 interacts with TERRA independently of the presence of Ter?

We agree with this important point, and now provide a satisfying explanation for these observations. Please see our response to Reviewer's 1 main point 1 and to Reviewer 2's point 4.

5) The demonstration that only TiTEL is elongated upon TERRA transcription is not completely clear. The authors mention in the text that the length of "the pure telomeric repeats" within TiTELs (as the nTELs) is around 200bp. However, by referring to fig S1b and S1c, the average length of TiTEL is about 300bp. Could the authors explain this discrepancy? Also in Fig S1c lane5, telomeres in the titel strain look more heterogenous (with shorter telomeres) compared to WT strain (compare lane 2 to lane 5). From the figures, it is not clear whether the TiTELs have the same telomere length than the nTELs. If TiTELs are shorter, it may that they will be preferentially elongated. This point needs to be clarified.

We believe that it is clear that tiTEL transcription induced by THI withdrawal does not promote telomere elongation of nTELs in trans. This is shown both by Southern blot analysis (Figure 4 A and B; Figure EVD) and by Telo-PCR sequencing (Figure EV3). As for whether tiTEL are shorter than nTELs, we show in Figure EV3D that indeed tiTELs are on average 30 nt shorter than nTELs possibly due to the fact that as long as cells are maintained in THI+ medium tiTERRA levels are low and within the levels of natural TERRA (Figure 3D). Nevertheless, tiTEL elongation occurs when transcription is induced.

6) Authors have deleted the trt1 to show that elongation of telomeres after TSA treatment and induction is telomerase-dependent. A slight elongation of telomere is still observed in presence of TSA. This elongation is not rad51 or exo1-dependant. Their conclusion is that TSA may provoke telomerase-independent elongation. Another possibility would be that senescence is delayed in presence of TSA thereby explaining the difference observed in telomere length in trt1 deleted strain. It would be helpful to understand how experience has been performed and to indicate at which stage of the senescence samples have been collected. It would have been preferable to use the tpz1K75A mutant strain that display short but stable telomeres.

This point is very well taken. Indeed, while THI did not affect cell growth, addition of TSA slowed down cell proliferation. All cells underwent ~ 8 population doublings in 24 hours in the absence of TSA regardless of *ter1+* status, while cells underwent only ~ 5 population doublings when TSA was present. We now clearly state this in the text and suggest that TSA alone could promote telomere elongation independently of telomerase or that replicative telomere shortening in TSA-treated *trt1*D cells occurred more slowly than in untreated counterparts (page 11). We also very much appreciate the suggestion of using the *tpz1K75A* mutant and we will establish tiTEL strains carrying that mutation for future studies.

Other points:
1) Figure 1C: It is surprising to observe that telomeric fragments run at an average of 400bp after Telo PCR. WT telomeric repeats run at approximately 300bp (as shown by Apa1 telobot Figure S1c lane 2).

We are very thankful to this Reviewer for spotting this inconsistency, because it brought to light an error in the marker molecular weights in the original Figure 1C. The correct size range goes from 1.2 to 0.7 kb and not from 0.6 to 0.2 kb as previously indicated (this is now corrected in Figure 1B). As for the size of the amplification products, this depends on the chosen subtelomeric oligonucleotide used for PCR. In this case we used the oligonucleotide oF5 (we have now indicated the identity of the oligonucleotide employed below the gels for clarity), which starts 627 bp upstream of the first telomeric repeat (oF5 sequence is shown in table EV2). The Telo-PCR products from wt cells are approximately 900 bp long, indicating that the pure telomeric tract is approximately 300 bp long.

2) The authors do not mention how do they perform senescence (liquid vs agar plate), and how they follow population doublings. Senescence curves might be informative.

Again many thanks for this important comment that allowed us to pinpoint an imprecision in the original manuscript. The correct number of population doublings are now incorporated into Figure

1B-D – in fact these cells underwent fewer pds than indicated in the original manuscript due to a mistake in the mathematical formula used. The corrected growth curves are now shown in Figure 1 B-D and the number of population doublings undergone by cells in the different experiments is now clearly described in the figures and text. We have also added a detailed description of how growth curves were generated in the Materials and Methods section (page 17).

3) In general, the MS is rather difficult to read. The figure legends need to be more detailed (for example Figure1). Also in Figure 3, lane numbers should be added to the figure and referred in the text.

We have thoroughly edited the manuscript for this revision and believe that it is now substantially improved in both content and presentation. We removed discussion paragraphs from the Results section and moved them to a newly introduced Discussion section, which we expect will improve the readability of the manuscript. As suggested, we also added lane numbers to the Southern blots shown in the current Figure 4A and B (originally Figure 3A and B) and we refer to them in the text (page 11). Lastly, we added more detailed information to all figure legends, as suggested.

2nd Editorial Decision 04 April 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees. Referees 2 and 3 still have a few more suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

Given that the character count of the manuscript largely exceeds the one of a typical short report in our journal, and given the rather busy figures, I would like to suggest to change the manuscript into a full article. The results and discussion sections are already separate, you would only need to add one more main figure. The model in figure 5 could for example be figure 6, or figure 1 could be split. Please also include the two tables in the methods section.

For all cropped gels, please always leave a white space between two cropped gel pieces. It is missing in figure EV1A, for example. Please also cite EV figures as "Fig EV1", etc in the manuscript text.

I look forward to seeing a new final version of your manuscript as soon as possible, and to seeing this work published.

REFEREE REPORTS

Referee #1:

The authors have included new data that answer the main comments of the reviewers. I think this manuscript is now acceptable for publication in Embo Reports.

Referee #2:

I have carefully read the revised manuscript of Moravec et al. Authors made a lot of additional experiments to address (most) reviewers' comments. Some experiments were successful while others were more disappointing.

One major concern that I still have deals with the association of TERRA with telomerase in conditions where telomere transcription is "physiologically" increased. After the first reading of the manuscript, I indeed suggested to check whether telomerase association with TERRA may be increased in ter1D cells. The authors performed the experiments but did not detect any enrichment after trt1-myc RIP (Fig. 2C). They concluded from their data that this may be explained by limiting telomerase levels in cells. However, in Fig. 5, authors show an enrichment of TERRA in trt1-myc RIP experiments performed in cells where telomere transcription was induced by removal of thiamine (Fig. 5C). Hence, the argument does not seem to be valid. What I noticed in Fig. 2C, was that TERRA transcription in that particular experiment with ter1D cells PD18 may not be that high, around 2.5-fold I would say (comparison between left and right panel). This may be the problem as, in Fig 5, tiTERRA polyA+ levels were increased by about 40-fold upon removal of thiamine.

Having said this, I would therefore suggest to either remove Fig 2C or to change the text accordingly and state that the absence of TERRA enrichment in RIP experiment may be due to the relatively modest increase in TERRA levels. But then, one would need to explain the discrepancies between Fig 2C and Fig 1D where, at PD18, polyA+ TERRA levels were induced by 20-40-fold.. In Fig 5C, this is also not mentioned on the graph whether RIP was normalized to input RNA. Based on the legend, it seems to be the case. Still, in these conditions of huge TERRA induction, normalizing to input RNA was not a problem and authors were able to detect an enrichment in RIP experiments. This further invalidates the experiment shown in Fig. 2C and the associated comments in the text. THIS NEEDS TO BE FIXED.

Minor comments:
-in Fig 2A, B and C, mentioning "input fraction" on top of the graph is misleading. I would rather mention the normalization to input fraction on the Y axis. This holds true for the rest of the manuscript.
-Fig 2B: showing a RIP experiment with DNaseI treatment without showing the efficiency of the treatment is not fully satisfying
-Fig. 2D is not mentioned in the text
-page 14: As long as a closeD state

Referee #3:

The revised version adequately satisfied my concerns. I do appreciate the efforts the authors have made to address all concerns. The conclusions has been strengthened by the additional experiments and the presentation of the manuscript has been really improved.

2 minors points :

- The calculation of population doublings in figure 1 is misleading. As the author started from a single colony for growth curve, they should take into account the approximate 25 pds that the est1- and ter1- cells went trough to form a colony (10+25 , 14+25 and 18+25). This should be modified in the figure, the legend and the text.

- In the model presented in figure 5E, the authors represented the Rap1 protein as a direct interactor

of telomeric repeated sequences. For a sake of consistency, it would be preferable that authors add Taz1 protein to their model (making the link between telomeric repeats and Rap1).

2nd Revision - authors' response 06 April 2016

Thank you very much for giving us the opportunity to submit a revised version of our manuscript entitled 'TERRA promotes telomerase-mediated telomere elongation in *Schizosaccharomyces pombe*' [Paper #EMBOR-2015-41708V2].

As you suggested, the cartoon sketching the model for TERRA-mediated activation/recruitment of telomerase at short telomeres is now shown as an independent figure (Figure 6). Hence, the revised manuscript comprises 6 main Figures, 4 Expanded View Figures and two Tables and can indeed be published as a full article. The total characters are approximately 46500 (with spaces) including the main text and Figure Legends but excluding References and Materials and Methods.

Moreover, to address your requests:
1) For all cropped gel membranes we have left white spaces between two cropped parts (see new Figure 3B and EV1A). Please also note that in the new Figure EV1B we now show a full membrane consecutively hybridized with nmt1 and telomeric probes. This replaces the cropped gel originally shown in panel B and the information previously provided by panels B and D is now consolidated in the current panel B. The relative figure legend has been changed accordingly.
2) We now cite all EV figures as "Fig EV" in the manuscript text.
3) We have added a short summary of the findings and their significance on page 2 of the text.
4) We have added 3 bullet points highlighting key results on page 2 of the text.
5) We are including a synopsis image (550x440 pixels) with a model for TERRA-mediated telomerase activation/recruitment at short telomeres.
6) We have moved the two tables to the Materials and Methods section and they are cited as Table 1 and 2 and not as Expanded View Tables.

Finally, please find below a detailed point-by-point response to the Reviewers' comments.

I hope that our manuscript is now acceptable for publication in *EMBO Reports* and I thank you once more for your precious help.

Point-by-point response to the Reviewers' comments

Referee #1:
The authors have included new data that answer the main comments of the reviewers. I think this manuscript is now acceptable for publication in Embo Reports.

Thank you very much for the constructive criticisms and for the support.

Referee #2:
I have carefully read the revised manuscript of Moravec et al. Authors made a lot of additional experiments to address (most) reviewers' comments. Some experiments were successful while others were more disappointing. One major concern that I still have deals with the association of TERRA with telomerase in conditions where telomere transcription is "physiologically" increased. After the first reading of the manuscript, I indeed suggested to check whether telomerase association with TERRA may be increased in ter1D cells. The authors performed the experiments but did not detect any enrichment after trt1-myc RIP (Fig. 2C). They concluded from their data that this may be explained by limiting telomerase levels in cells. However, in Fig. 5, authors show an enrichment of TERRA in trt1-myc RIP experiments performed in cells where telomere transcription was induced by removal of thiamine (Fig. 5C). Hence, the argument does not seem to be valid. What I noticed in Fig. 2C, was that TERRA transcription in that particular experiment with ter1D cells PD18 may not be that high, around 2.5-fold I would say (comparison between left and right panel). This may be the problem as, in Fig 5, tiTERRA polyA+ levels were increased by about 40-fold upon removal of thiamine. Having said this, I would therefore suggest to either remove Fig 2C or to change the text accordingly and state that the absence of TERRA enrichment in RIP experiment may be due to the

relatively modest increase in TERRA levels. But then, one would need to explain the discrepancies between Fig 2C and Fig 1D where, at PD18, polyA+ TERRA levels were induced by 20-40-fold.. In Fig 5C, this is also not mentioned on the graph whether RIP was normalized to input RNA. Based on the legend, it seems to be the case. Still, in these conditions of huge TERRA induction, normalizing to input RNA was not a problem and authors were able to detect an enrichment in RIP experiments. This further invalidates the experiment shown in Fig. 2C and the associated comments in the text. THIS NEEDS TO BE FIXED.

Thank you very much for this thorough analysis of our RIP experiments using *ter1*+ deleted strains. However, we are convinced that the results presented in the aforementioned figures do not invalidate each other. It is true that in the *ter1*D strain used in Figure 2C TERRA induction is not as high as in the ones used in Figure 1D and we believe that this is due to the fact that in Trt1-myc-tagged strains, telomeres, although properly maintained and functional, are shorter than in untagged wt cells (see original paper describing the strain: Tang W, Kannan R, Blanchette M, Baumann P (2012) *Nature* 484: 260-4). TERRA levels in Trt1-myc strains are therefore expected to be higher than in untagged wt strains and as a consequence TERRA induction upon deletion of *ter1*+ is likely to be more modest when levels are compared between Trt1-myc *ter1*+ and Trt1-myc *ter1*D cells. We now mention this point in the text (page 8). Nevertheless, results obtained with tiTEL strains do not invalidate our hypothesis that telomerase levels in cells are limiting, but rather support it. While in Figure 2 A-C we are analyzing total TERRA generated from at least 4 out of 6 *pombe* subtelomeres (we cannot design oligonucleotides discriminating between subtelomeres of different chromosome ends as they are identical), in Figure 5C we are specifically looking at tiTERRA, i.e. TERRA produced from one subtelomere only. The fact that we detect increased amounts of tiTERRA pulled down with Trt1-myc when transcription is induced (THI-) indicates that we are 'funneling' telomerase molecules towards interaction with one specific TERRA pool (in this case tiTERRA) and disfavoring interaction with endogenous TERRA from other chromosome ends; this *per se* supports the hypothesis that telomerase is limiting. Further strengthening this idea, TSA treatments of tiTEL strains in induced conditions (THI- TSA+) lead to a ~ 7 fold increase in polyA+ tiTERRA interaction with Trt1-myc (as compared to THI+ TSA- samples, see Figure 5C), while in the same conditions total polyA+ tiTERRA levels increase by hundreds of folds. We have better explained this important point in the text by stating that 'We conclude that increasing tiTEL transcription leads to a preferential binding of telomerase to tiTERRA, likely disfavoring binding to natural TERRA from other telomeres' (page 13). We believe that Figure 2C adds valuable information to our study and we have therefore decided not to remove it.

Minor comments:
-in Fig 2A, B and C, mentioning "input fraction" on top of the graph is misleading. I would rather mention the normalization to input fraction on the Y axis. This holds true for the rest of the manuscript.

We have removed 'input fraction' from the top of the graphs and indicated it on the y axis. Moreover, how values are expressed is clearly indicated in all figure legends.

-Fig 2B: showing a RIP experiment with DNaseI treatment without showing the efficiency of the treatment is not fully satisfying

DNase treatments were performed directly on beads (thus on very limited amounts of nucleic acids) using 30 units of DNaseI per sample and incubating at 30 °C for 1 hour. In these conditions, DNaseI completely digests more than 10 micrograms of genomic DNA.

-Fig. 2D is not mentioned in the text

Thank you for noticing this. We now mention that '*ter1*+ deletion did not affect the levels of Trt1-myc in cells' and reference the figure panel (page 8).

-page 14: As long as a closeD state

This has been corrected.

Referee #3:
The revised version adequately satisfied my concerns. I do appreciate the efforts the authors have made to address all concerns. The conclusions has been strengthened by the additional experiments and the presentation of the manuscript has been really improved.

Thank you very much for the constructive criticisms and for the support.

2 minors points :
- The calculation of population doublings in figure 1 is misleading. As the author started from a single colony for growth curve, they should take into account the approximate 25 pds that the est1- and ter1- cells went trough to form a colony (10+25 , 14+25 and 18+25). This should be modified in the figure, the legend and the text.

This is absolutely true. We now indicate that 'considering that transformed cells underwent approximately 25 pds on plate, the final number of pds are estimated to be ~ 25+10 (~ 35), ~ 25+14 (~ 39) and ~ 25+18 (~ 43)'. This is stated on page 5 of the text, clearly indicated in Figure 1 B-D and relative legend, and in the Materials and Methods section.

- In the model presented in figure 5E, the authors represented the Rap1 protein as a direct interactor of telomeric repeated sequences. For a sake of consistency, it would be preferable that authors add Taz1 protein to their model (making the link between telomeric repeats and Rap1).

We have added Taz1 to the model (Figure 6) and we mention in the Discussion that loss of Rap1 from telomeres undergoing shortening occurs as a consequence of Taz1 release (page 15).

3rd Editorial Decision 07 April 2016

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Claus M. Azzalin
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41708V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All experiments were repeated at least three times and the exact number of repetitions is indicated in figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	na
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample was excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization procedure was used.
For animal studies, include a statement about randomization even if no randomization was used.	na
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No (most experiments are molecular biology)
4.b. For animal studies, include a statement about blinding even if no blinding was done	na
5. For every figure, are statistical tests justified as appropriate?	We have always used a Student's t-test (indicated in each figure legend).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Except for telomere sequencing experiments, the sample size is generally small (n≤5). Although this number is low, Student's t-test should be considered acceptable.
Is there an estimate of variation within each group of data?	Yes, SD are always shown.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All utilized antibodies are commercially available and cat. numbers are inidcated in the Material and Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yeast strain culture and generation are indicated in the Material and Methods section.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	na
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	na
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	na

E- Human Subjects

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

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	PacBio sequences were deposited at the Sequence Read Archive (SRA; accession number: PRJNA307606). This is indicated in the Material and Methods section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	na
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	na
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	na
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	na

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No