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DNA-end capping by the budding yeast transcription factor and subtelomeric binding protein Tbf1

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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 06 April 2011

I am herewith sending you the referee reports on your recent Tbf1 manuscript. Please apologize the slight delay in providing you with a decision, but due to the somewhat ambivalent evaluations and my being away from the office it took us somewhat longer here to reach a final conclusion on the potential suitability of the study for publication in The EMBO Journal. While referee 1 is generally supportive of publication (pending clarification of one major concern), referees 2 and 3 both raise a number of substantive points that would in their opinion need to be addressed to make the study more conclusive. After careful assessment of these various criticisms, we decided that we should be able here to consider a revised manuscript further for publication; however, even though we feel that certain referee requests are not essential for such a revision, it is apparent that the number of experimental issues that need to be addressed is still substantial, and we would thus also understand if you were to decide to publish the study rapidly and without major changes elsewhere.

In case you would like to revise the manuscript for The EMBO Journal, the key issues that will be important to address are:
- the quantification of the ChIP data, as particularly detailed by referee 1
- better discussion of the models on Tbf1 ancestral/present telomere functions (ref 2 points 2, 4)
- extending the checkpoint arrest data (ref 2 point 6)
- testing Tbf1 function on endogenous (sub-)telomeres, irrespective of the outcome (ref 3 point 1)
- the more specific/control issues 3-8 of referee 3
- ref 3 point 10 on caveat from fission yeast telomeres needs to be discussed

On the other hand, several other points may be beyond the scope of current analysis, and addressing

them in writing would be sufficient of you should not be able to do so experimentally:
- ref 2 point 1 on Tbf1 mechanism on short and long repeats
- the use of native rather than HO-induced humanized telomeres (referee 2 points 3, 5)
- telomerase recruitment questions (ref 2 points 7, 8)
- ref 3 point 2

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. I should remind you that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version; we would in this case however be open to extending the revision period of normally three months to up to six months should you require this. In any case, please do not hesitate to contact me should you have any questions related to this decision and your revision

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this paper, Shore and co-workers elucidate regulatory mechanisms of how chromosome ends which contain Tbf1-binding sites but lack Rap1-binding sites are capped and can be maintained by the telomerase enzyme. The work indicates that Tbf1 can replace Rap1 function at telomeres for telomere capping and telomerase recruitment. Surprisingly, the paper also discovers that telomerase binds to DNA breaks, though they are not healed by telomere addition. This work contributes to the understanding of telomerase regulation and telomere capping. The comparison of the Rap1 versus Tbf1 maintenance mechanisms is of broad general interest. I find the paper to be well done but I suggest that the data should be quantified in a different manner as argued below.

Major point:

Quantification of ChIP data: The ChiP signal is indicated as fold enrichment over the background. Although this has been done by others in the field before, I don't think this the best way to express the data. The background signal should in an ideal world not exist. Thus, I find it odd to use this signal for normalization. The fraction of telomeric DNA or other DNA that is pulled down would be more informative. This becomes especially important when comparing binding of telomerase and Cdc13p to telomeres versus other DNA ends as done in Figure 3 and elsewhere. If the background signals are very different for the two DNA sequences, the fold enrichment over background does not indicate the true relative abundance of telomerase or Cdc13p at these ends. Thus, from the current data, it is in my view not possible to compare binding strengths of telomerase (or Cdc13p) to telomeres and DNA double strand breaks.

Other points:

Rap1 binding as a measure for telomere elongation: I suspect this read-out has been validated. The authors can give a reference for this or indicate how they have verified this relation.

I don't find the title very clear nor very appealing.

Referee #2

Number-dependent DNA-end capping by the budding yeast Tbf1 protein

The authors examined the function of Tbf1 at induce double strand break (DSB) strain. These DSB sites were induced with HO and had either a short (60bp) human telomeric sequence or a long (230bp) human telomeric sequence adjacent to the break. With a combination of different ChIP experiments to short and long T2AG3 arrays they tested the model that Tbf1 acts directly in end protection. They were able to show that Tbf1 binds short and long T2AG3repeats but that only the short repeats were elongated after inducing of the break. This elongation is dependent on Tbf1 association, since mutation of Tbf1 results in equal elongation of long and short repeats. In contrast to short repeats, which were recognized as DNA damage and induce a G2/M checkpoint arrest, long repeats were not seen as DSB and do not activate a checkpoint arrest. They demonstrated with Est2 and Est1 ChIP that similar levels of telomerase are associated to short and long repeats. They concluded that Tbf1, similar to Rap1, regulates telomerase recruitment and activity at long and short repeats and that the MRX complex is directly involved in telomerase recruitment to short repeats.

Overall, they representative of a lot of work. The data seem to be well-controlled, and the results are certainly potentially interesting, but there are a few issues of concern:

1. If, as the authors propose, Tbf1 is required to protect T2AG3 metazoan repeats and helps to distinguish between long and short repeat, how is Tbf1 mechanism at these repeats? How many Tbf1 proteins are necessary to bind/ block elongation?

2. If their model and take home message is that Tbf1 is descended from an ancestral telomere-binding protein, then it seems like the cited papers and the overall model deserves more of a discussion than a simple throwaway reference at the end of the paper. It would be nice to expend that part of the discussion, and explain more about its implications for recruitment/action of telomerase to T2AG3 ends?
They report that they data provide additional strong evidence to the model that Tbf1 is an ancestral telomere binding protein. I agree that their data support the current model, but it would be great to see if the new identified function is conserved through evolution.

3. Furthermore it would great to see if T2AG3 telomeres (native telomeres) are maintained through the same mechanism as telomeric repeats after HO induction. They could use a alternative telomerase RNA to generate such telomeres, as it has been done by (Alexander & Zakian, Brevet et al 2003, Henning et al 1998) and use an induced short telomere strain.

4. It would be interesting to discuss the function of Tbf1 in S. cerevisiae with the outcome of these experiments. The tested repeat is a humanized telomeric repeat and not found native in yeast cells. Can the function of Tbf1 at subtelomeric regions be explained by the new data? Is Tbf1 involved in protecting breaks in subtelomeric regions or internal regions?

5. It would be interesting to see how long/short native telomeres are in the experimental strains. Are the native telomeres also long if the repeat is long? It would be great if we know what happens at native telomeres in the experiments. The tested mutants are known telomere binding proteins with a strong phenotype. Are the telomere binding proteins recruited away from telomeres after induction of HO and by this shifting the equilibrium at telomeres. What is the behavior of the tested proteins in these experiments at native telomeres ? Are the enrichment of binding as high as published data?

6. The Checkpoint arrest data are very interesting and provides a strong support for their model. It would be interesting if they could extend this assay (Fig 2A) by measuring the % of budded cells in tbf1- Δi, and also in cdc13-2, mre11Δ, mec1Δ and tlc1Δ-48 cells. Furthermore, it would be nice to check if the cells are arrested or if they are dying and therefore not released into the cell cycle. These additional experiments will provide better inside into the mechanism of telomerase recruitment checkpoint activation and Tbf1 function.

7. Their experiments clearly show that telomerase is associated to long and short repeats and that their activity is regulated by Tbf1. With ChIP and qPCR they attempted to address the question how telomerase is recruited to these sites. However all of their tested candidates, Cdc13, Ku70/80, and

Tel1 had no impact on telomerase binding but a small impact on telomerase activity at short repeats. However it would be very beneficial for the reader if the question how telomerase is recruited and activated will be answered.

8. Its very interesting result that telomerase is recruited to a centromere less chromosome fragment. But why would telomerase be recruited to these fragments? Do the authors think there is something special about this site, or do they propose that telomerase would bind at this same level to the centromere-proximal side of a DSB lacking TG sequences? That is, do they think that this site is representative of any type of DSB lacking TG repeats?

Minor comment

1. Many error bars are very large and it would be interesting to see statistics if the difference is really significant. For example Fig 6A,B or 7.

2. It would be very beneficial, if the authors would explain why they normalized all their ChIP signal to PDI1.

3. In the last paragraph on p7 they presents the data to Fig 2A. They show that long T2AG3 repeats have no checkpoint response and are not seen as DSB they say 97.6% survived induction. Short repeats have a reduced survival rate 83%. The authors claim that this is significant. Is this confirmed by any statistical analysis?

4. They tagged Est1 and Est2 in the absence of Mec1 and analyzed their binding by ChIP. Mec1 deficient cells are only viable together with deletion of Sml1. A better control than WT for this ChIP experiments would be a sml1Δ strain.

5. According to Fig 1 it seems like that VII-L was not cut as well as V-R. Is there a reason for this difference?
In addition it seems like, although they have a loading control on each panel to control for the cleavage, their loading is uneven so it is very hard to interpret these results. They normalized to the HO cutting, but I am not sure there is a 1:1 ratio between DSB cuts and the relative enrichment in ChIP.
Furthermore it would be interesting to quantify these data in a clearer way to also compare them with each other.

6. I am not sure that for me the equation, described in methods p20 in the ChIP protocol is clear. Do they mean that they multiplied the AMP Ab+ by the fraction of HO cut, rather than multiplying AMP Ab+ by 1 over the percentage of HO cut?

Referee #3

Manuscript Number: EMBOJ-2011-77482
Title: Number-dependent DNA-end capping by the budding yeast Tbf1 protein
Corresponding Author: Prof. Shore

Budding yeast cells normally contain TG repeats at telomeres but can use human telomeric TTAGGG repeats as telomere sequence. Rap1 protein binds to endogenous TG telomeres whereas Tbf1 covers telomeric TTAGGG repeats (Alexander and Zakian, 2003 EMBO J, 1688-1696). In this paper the authors have studied how budding yeast cells maintain TTAGGG repeats as part of telomere.

From the data provided, the authors proposed that Tbf1 down-regulates telomere extension and checkpoint activation. Tbf1 inhibits the recruitment of Mre11-Rad50-Xrs2 complex to telomeres. However, the Tbf1-mediated effect is observed only if a 230 bp TTAGGG array is artificially placed at a DNA end. There is no such long TTAGGG repeat on chromosomes in budding yeast cells. It thus remains unclear that the Tbf1-dependent function operates normally in wild-type cells. In other

words, this manuscript does not provide evidence that the proposed Tbf1 function is physiologically relevant. It should be noted that fission yeast Tbf1 does not appear to play a key role in telomere maintenance (Cockell et al., 2009. Eucaryotic Cell 207-216).

Specific Points

1. The authors should show that loss-of-function in TBF1 results in telomere elongation or checkpoint activation in cells carrying endogenous sub-telomeres and telomeres. This would be one of key experiments to determine whether the proposed Tbf1 function is physiologically relevant.

2. The authors argued that like Rap1, Tbf1 contributes specifically to telomere protection because other transcription factors (Bas1, Abf1, Reb1) did not protect DNA ends from degradation. The respective constructs contained binding site arrays of 9 or 24 tandem copies for each of these transcription factors. The 230 TAG repeat sequence may contain more than 30 copies of TTAGGG because TTAGGG is only 6 bp long. The authors need to show that Tbf1 effectively protects DNA ends if 24 TTAGGG repeats are placed nearby.

3. Cdc13 binding protects telomeres from DNA degradation; that is, Cdc13 acts as a telomere cap. Cdc13 binds TTAGGG repeats as well as TG repeats (Alexander and Zakian, 2003 EMBO J, 1688-1696). The authors should examine whether Cdc13 binds the Bas1, Abf1 or Reb1 repeats after HO induction. If Cdc13 does not bind these repeats, the provided data do not support the idea that Tbf1 protects TTAGGG ends. The current data do not exclude the possibility that Cdc13 plays a key role in TTAGGG end protection.

4. In Figure 1, Cdc13 binding data should be included. Because the authors argue that Cdc13 binding is critical for telomere addition, the tbf1-deletion-i mutation could affect Cdc13 binding.

5. In Figure 2, the authors need to confirm DSB induction by Southern blotting.
It is not clear whether HO expression indeed generates a DNA break into the cleavage site at the 230 bp TAG-CTA repeats. If the sequence is resistant to HO cleavage, it is not surprising that HO expression does not activate checkpoint.

6. Does the tbf1-deletion-i mutation affect the expression level of Est1 or Est2 in Figure 3? It is curious that Est1 or Est2 associates with DSBs poorly in tbf1-deletion-i cells.

7. Southern blots, which monitor telomere addition, should be included in Figure 6. There is so far no evidence that Rap1 binding is correlated to telomere addition.

8. Figure 7. Viability in wt and mec1 deleted cells after HO induction should be included to support telomere addition at DSB ends in mec1 deletion mutants. The authors need to confirm telomere addition by Southern blotting.

9. There is no figure legend for Fig. S5. It is impossible to evaluate the data.

10. Telomeres of fission yeast consist of long TTAGGG repeats. The fission yeast Tbf1 protein binds telomeric repeat TTAGGG but does not appear to protect telomere ends from checkpoint activation or play a key role in telomere maintenance. The authors need to discuss the difference between budding and fission yeast Tbf1 functions in "Conclusions and implications for telomere evolution".

Minor points

1. The detailed culture condition should be included in the figure legend, because the 230 bp TAG sequence became shorter in Fig. 4 and Fig. 5 but did not in Fig. 1.

2. Fig, S5 (page 13, line 5) should be Fig, S4.

1st Revision - authors' response 14 July 2011

We would like to thank you again, as well as the referees, for your time and effort in evaluating our manuscript. As detailed below, we have responded to every single comment and/or suggestion of the three referees. In many cases this has resulted in us performing additional experiments (results of which are presented in 7 new figures for the referees, as well as 3 completely new Supplementary Figures and 1 very extensively revised Supplementary Figure, containing a large amount of new data). We have also carefully evaluated and validated the statistical significance of all of the specific qPCR ChIP data questioned by the reviewers. We think that this was a particularly useful exercise and thank the referees for asking us to present these data in the manuscript (many of which we already had in hand).

Perhaps the most significant critique was raised by Reviewer #3, who questioned the physiological significance of our results with regard to the possible telomeric function of Tbf1 in wild type yeast cells. Looking back at our original manuscript we realized that indeed we had not dealt with this issue carefully enough. In the revision we explicitly address this point near the end of the Discussion, spelling out how data in this paper and in previous publications (one of which we had failed to cite previously) point strongly to a telomeric role for Tbf1. More importantly, though, the reviewer's comment motivated us to look more carefully at this issue and to perform two new experiments. First, we showed that a short internal T_2AG_3 tract can very strongly promote healing at a TG-11bp end that is itself too short to seed telomere formation (Supplemental Fig. 8). This is a very clear indication that native subtelomeric Tbf1 may be able to rescue critically short telomeres *in vivo* in wild type cells. We also looked more carefully at the *tbf1*-D*I* mutant and showed, remarkably, that it causes a telomere elongation phenotype (Supplemental Figure 7). This is a very striking result, particularly when one realizes that a hypomorphic (DAmP) allele of *TBF1* causes telomere shortening (Ungar et al. (2009) *NAR*). Significantly, both of these *TBF1* alleles cause a slow growth phenotype. This situation, where different alleles of a gene have opposite effects on telomere length, is remarkably similar to that of *RAP1*, the primary telomere-repeat binding protein in budding yeast, and argues very strongly for a direct role of Tbf1 in telomere biology in this yeast.

Taking into account these and all of the other points described in detail below, we believe that we have now positively responded to all of the comments of the reviewers, and in the process considerably improved our manuscript. We hope that you and the referees will agree, and that the manuscript will be quickly approved for publication in the *The EMBO Journal*.

We are at your complete disposal for any questions that might arise, or any additional information that you feel might be required. Finally, we would like to thank you once again for your time and effort on our behalf.

Dear Referee #1

Major point:

Quantification of ChIP data: The ChiP signal is indicated as fold enrichment over the background. Although this has been done by others in the field before, I don't think this the best way to express the data. The background signal should in an ideal world not exist. Thus, I find it odd to use this signal for normalization. The fraction of telomeric DNA or other DNA that is pulled down would be more informative. This becomes especially important when comparing binding of telomerase and Cdc13p to telomeres versus other DNA ends as done in Figure 3 and elsewhere. If the background signals are very different for the two DNA sequences, the fold enrichment over background does not indicate the true relative abundance of telomerase or Cdc13p at these ends. Thus, from the current data, it is in my view not possible to compare binding strengths of telomerase (or Cdc13p) to telomeres and DNA double strand breaks.

The reviewer raises a good point, one that we believe is not resolved in the field. Although we have reported our ChIP data as "fold enrichment" relative to a sequence not expected to be bound specifically by the protein in question, which is common practice in the field, we have also measured IP input DNA in all of our experiments. and thus have also evaluated our IP data as a

fraction in input DNA. In no case did we find a significant difference in the outcome, and thus we feel confident in the validity of our conclusions (see appended **Fig. R1** and compare to Fig. 3 and Fig. 7C). It is also important to realize that we compare binding of Cdc13 and telomerase to telomeric and non-telomeric (DSB) ends in the same strain, normalizing both to the same control locus (*PDI1*). We thus believe that the TG to non-TG comparison is equally valid regardless of how one calculates the binding (percent IP versus fold enrichment), and this is indeed what our data show.

Other points:

Rap1 binding as a measure for telomere elongation: I suspect this read-out has been validated. The authors can give a reference for this or indicate how they have verified this relation.

The referee is correct: this has been validated by Hirano & Sugimoto (2009) *Mol Cell*, and more recently by Zhou et al. (2011) *PLoS Genetics*. We now note this in the manuscript.

I don't find the title very clear nor very appealing.

Upon reflection, we think that the referee is absolutely correct, and we thank he/she for inciting us to come up with something better. We have changed the title to read: "DNA-end capping by the budding yeast transcription factor and subtelomeric binding protein Tbf1" (comments/suggestions still welcome).

Referee #2

1. If, as the authors propose, Tbf1 is required to protect T2AG3 metazoan repeats and helps to distinguish between long and short repeat, how is Tbf1 mechanism at these repeats? How many Tbf1 proteins are necessary to bind/ block elongation?

This is an interesting question. We have thus examined T_2AG_3 arrays of 120 and 200 bp and find that neither block elongation by telomerase at an HO break (please see appended **Fig. R2**). We thus conclude that somewhere between 200 and 230 bp of T_2AG_3 repeat are required to efficiently block detectable telomerase action in the 4 hours following HO induction.

2. If their model and take home message is that Tbf1 is descended from an ancestral telomere-binding protein, then it seems like the cited papers and the overall model deserves more of a discussion than a simple throwaway reference at the end of the paper. It would be nice to expend that part of the discussion, and explain more about its implications for recruitment/action of telomerase to T2AG3 ends?

We agree, and have now expanded our discussion of this issue.

They report that they data provide additional strong evidence to the model that Tbf1 is an ancestral telomere binding protein. I agree that their data support the current model, but it would be great to see if the new identified function is conserved through evolution.

We agree with the referee that this is an interesting issue, but to address this experimentally in a serious way would be well beyond the scope of the present study.

3. Furthermore it would great to see if T2AG3 telomeres (native telomeres) are maintained through the same mechanism as telomeric repeats after HO induction. They could use a alternative telomerase RNA to generate such telomeres, as it has been done by (Alexander & Zakian, Brevet et al 2003, Henning et al 1998) and use an induced short telomere strain.

We believe that this has already been demonstrated by Alexander & Zakian (2003. *EMBO J.* **22** 1688-96), by the generation of a length-regulated "all T_2AG_3 repeat" telomere through a homologous recombination - telomere healing strategy. There is no reason to believe that telomere healing at a T_2AG_3-repeat end generated by HO would be any different. Similarly, an induced short telomere in a strain containing an alternative ("humanized") telomerase would be expected to

elongate to a roughly set average length (Alexander & Zakian, 2003). We would thus argue that this elaborate experiment, which would take a considerable effort to set up, would only provide an additional confirmation of what we already know, and would tell us nothing new. Furthermore, understanding the molecular mechanisms driving and regulating T_2AG_3 repeat using an induced short telomere strain would require an enormous additional investment, and would clearly be beyond the scope of the present study.

4. It would be interesting to discuss the function of Tbf1 in S. cerevisiae with the outcome of these experiments. The tested repeat is a humanized telomeric repeat and not found native in yeast cells. Can the function of Tbf1 at subtelomeric regions be explained by the new data? Is Tbf1 involved in protecting breaks in subtelomeric regions or internal regions?

This is a very important point, and the referee's comments indicate that we have not been clear enough about this in our manuscript. The human (vertebrate) T_2AG_3 repeats are indeed found (in a somewhat dispersed form) at all yeast telomeres, within the conserved subtelomeric X elements and at Y' junctions. In a previous study from our lab (Preti et al. (2010) *Molecular Cell*) we reported a ChIP-seq experiment that detected very high levels of Tbf1 binding at nearly all telomeres. This fact, combined with our HO-cut healing experiments (discussed in detail below), indeed suggests exactly what the referee proposes, namely that Tbf1 is involved in protecting (and healing) telomeres that have undergone extensive TG repeat loss, for example due to replication fork collapse at or near the repeats. The results of Arenic et al. (2007) *EMBO reports* ("Tel1 kinase and subtelomere-bound Tbf1 mediate preferential elongation of short telomeres by telomerase in yeast") also strongly support this idea.

5. It would be interesting to see how long/short native telomeres are in the experimental strains. Are the native telomeres also long if the repeat is long? It would be great if we know what happens at native telomeres in the experiments. The tested mutants are known telomere binding proteins with a strong phenotype. Are the telomere binding proteins recruited away from telomeres after induction of HO and by this shifting the equilibrium at telomeres. What is the behavior of the tested proteins in these experiments at native telomeres ? Are the enrichment of binding as high as published data?

We have now measured telomere length for all native telomeres with Y' ends (about half of all telomeres in our haploid strains) and show that they are unchanged (attached **Fig. R3**). This result is not unexpected, since there are 32 native telomeres and we induce a single HO break in these strains. Titration effects are thus not likely to be significant.

6. The Checkpoint arrest data are very interesting and provides a strong support for their model. It would be interesting if they could extend this assay (Fig 2A) by measuring the % of budded cells in tbf1- Di, and also in cdc13-2, mre11D, mec1 D and tlc1 D-48 cells. Furthermore, it would be nice to check if the cells are arrested or if they are dying and therefore not released into the cell cycle. These additional experiments will provide better inside into the mechanism of telomerase recruitment checkpoint activation and Tbf1 function.

The reviewer raises a good point here. We have thus performed the cell cycle arrest assay for *tbf1*-D*i*, *cdc13-2, tlc1*-D*48* and *mre11*-D mutants, for both long and short T_2AG_3 repeat ends (see new Fig. S2). We also measured survival (% viability) in all of these contexts, and have incorporated these data into a **revised Fig. S2**). Significantly, neither of the telomerase pathway mutations (*cdc13-2* or *tlc1*-D*48*) had an effect on arrest when the HO cut was flanked by long T_2AG_3 repeats, though they perhaps had a very minor effect on survival (Fig. S2). These data clearly show that neither telomerase recruitment pathway is required for capping 230 bp T_2AG_3 tracts. As expected, both pathways contribute to capping at the short T_2AG_3 repeats (Fig. S2C), as well as survival (Fig. S2E), though neither do so to the same extent as Mre11. We also examined the *tbf1*-D*i* mutant and showed that it causes a short (0.5 hrs) but significant delay at long tracts, and displays an arrest phenotype at short tracts as well as a drop in viability, comparable to that observed in the *vid22 ygr071c* double mutant (**Fig. S2**). These new data are introduced into the text at the end of the section entitled: "Short T_2AG_3 arrays induce a transient G2/M arrest, but long arrays are capped" (pg. 9).

7. Their experiments clearly show that telomerase is associated to long and short repeats and that their activity is regulated by Tbf1. With ChIP and qPCR they attempted to address the question how

telomerase is recruited to these sites. However all of their tested candidates, Cdc13, Ku70/80, and Tel1 had no impact on telomerase binding but a small impact on telomerase activity at short repeats. However it would be very beneficial for the reader if the question how telomerase is recruited and activated will be answered.

This is a valid point that we have tried to address experimentally. Unfortunately, we have been unable to obtain Yku mutants in combination with either *cdc13-2* or *tel1-D* in our GAL-HO strains, presumably due to synthetic lethality. Our data suggest that the two telomerase recruitment pathways are redundant at these ends.

8. Its very interesting result that telomerase is recruited to a centromere less chromosome fragment. But why would telomerase be recruited to these fragments? Do the authors think there is something special about this site, or do they propose that telomerase would bind at this same level to the centromere-proximal side of a DSB lacking TG sequences? That is, do they think that this site is representative of any type of DSB lacking TG repeats?

These are good questions. No, we do not think that the centromere-less end is special in this regard, and this is reinforced by our own experiments (data not shown and Ribeyre et al. submitted) as well as by published data from other groups (Oza et al. (2009) *Genes & Dev*; Chung et al. (2010) *PLoS Genetics*) showing that telomerase is recruited to non-TG ends in general.

Minor comment

1. Many error bars are very large and it would be interesting to see statistics if the difference is really significant. For example Fig 6A,B or 7.

This is indeed a very valid point, and we thank the referee for pointing this out. We have now done what we believe is the correct test (Mann-Whitney) to evaluate statistical significance for these sorts of data sets. For Fig. 6A, the increase in telomerase recruitment at both short tracts and the DSB in the *yku70-D* mutant, relative to wild type, is significant ($p<0.05$). Similar comparisons for the other mutants show no significance. With regard to telomere elongation (as measured by Rap1 binding, Fig. 6B) all mutants show a significant difference compared to wild type at 4 hrs. (and *cdc13-2* already at 2 hrs.). For Fig. 7B, only the difference between WT and *mec1* for the DSB at 3 and 4 hrs. is significant ($p=0.03$ and $p=0.028$, respectively). These statistical analyses are now included in the relevant figure legends.

2. It would be very beneficial, if the authors would explain why they normalized all their ChIP signal to PDI1.

As explained above, *PDI1* is a non-telomeric (chromosome internal) site where binding of telomere or DNA damage-specific proteins is not expected to occur. This is a standard approach to evaluate "fold enrichment" at a specific site, but as we point out above, a measurement of percent IP vs. input (which we have also done) does not alter our conclusions.

3. In the last paragraph on p7 they presents the data to Fig 2A. They show that long T2AG3 repeats have no checkpoint response and are not seen as DSB they say 97.6% survived induction. Short repeats have a reduced survival rate 83%. The authors claim that this is significant. Is this confirmed by any statistical analysis?

This is indeed confirmed by statistical analysis.

4. They tagged Est1 and Est2 in the absence of Mec1 and analyzed their binding by ChIP. Mec1 deficient cells are only viable together with deletion of Sml1. A better control than WT for this ChIP experiments would be a sml1-D strain.

The reviewer is correct, and we have thus done this control. As expected, the same pattern is observed (see **Fig. R4**).

5. According to Fig 1 it seems like that VII-L was not cut as well as V-R. Is there a reason for this difference? In addition it seems like, although they have a loading control on each panel to control for the cleavage, their loading is uneven so it is very hard to interpret these results. They normalized to the HO cutting, but I am not sure there is a 1:1 ratio between DSB cuts and the relative enrichment in ChIP. Furthermore it would be interesting to quantify these data in a clearer way to also compare them with each other.

The reviewer is correct to note that there are differences in HO cutting efficiency between different constructs (perhaps related to the precise sequence context around the HO site and consequent effects on nucleosome positioning). That is why we have been very careful to measure the cutting efficiency in every single experiment, using an internal control sequence to correct for loading variations. Normalizing the measured ChIP enrichment to these values for cutting efficiency should give a precise measure of protein binding per DNA end. We note that this careful analysis goes well beyond the standard in the field (no measure of cutting efficiency at all).

6. I am not sure that for me the equation, described in methods p20 in the ChIP protocol is clear. Do they mean that they multiplied the AMP Ab+ by the fraction of HO cut, rather than multiplying AMP Ab+ by 1 over the percentage of HO cut?

We thank the reviewer for pointing this out. An error had indeed slipped into the equation presented, which is now corrected and written out in a way that more explicitly indicates how the actual Ct data from the qPCR measurements is treated to arrive at a "fold-enrichment" value.

Referee #3

*From the data provided, the authors proposed that Tbf1 down-regulates telomere extension and checkpoint activation. Tbf1 inhibits the recruitment of Mre11-Rad50-Xrs2 complex to telomeres. However, the Tbf1-mediated effect is observed only if a 230 bp TTAGGG array is artificially placed at a DNA end. There is no such long TTAGGG repeat on chromosomes in budding yeast cells. It thus remains unclear that the Tbf1-dependent function operates normally in wild-type cells. In other words, this manuscript does not provide evidence that the proposed Tbf1 function is physiologically relevant. It should be noted that fission yeast Tbf1 does not appear to play a key role in telomere maintenance (Cockell et al., 2009. Eukaryotic Cell **8**: 207-216).*

We thank the reviewer for pointing out the importance of physiological relevance regarding the capping and telomere healing functions of Tbf1 described here. Indeed, we did not explain in the original manuscript why we think this had been demonstrated, both through our own work and that published recently by others, as detailed below. In addition, we have gathered further evidence for a physiological role of Tbf1 in normal telomere maintenance, through two types of experiments. Our response to the specific comments of the reviewer, as well as a summary of our new data and arguments relevant to this point are as follows:

(i) To begin with, a quite significant capping and telomere healing effect is observed in our experiments with the short T_2AG_3 repeats (Fig. 2 and Fig. S2): note specifically the Tbf1- and Vid22/Ygr071-dependent survival frequency for 60 bp T_2AG_3 ends, many orders of magnitude higher than for DSBs. Our ChIP-seq analysis of Tbf1 binding (Preti et al. (2010) *Molecular Cell,* and data not shown) demonstrates very strong binding of Tbf1 at subtelomeric regions even where perfect T_2AG_3 repeats are not evident. We thus think that the absence of extremely long (230 bp T_2AG_3 arrays) at native telomeres is of no particular significance, and that Tbf1 bound to subtelomeric sites in wild-type cells may indeed carry out important "backup" functions in telomere length regulation and healing (see below).

(ii) Relevant to the latter point, we have now performed an experiment in which we test the ability of a short T_2AG_3 array to promote telomere healing at an 11 bp TG-repeat sequence that itself is too short to either cap or promote *de novo* telomere formation at a DNA end. This situation mimics that of a critical telomeric DSB in which too little TG repeat remains for efficient rescue. This experiment (now mentioned in the Discussion and incorporated into Supplementary Information, see **Fig. S9**) clearly shows that internal 60 bp T_2AG_3 arrays can strongly stimulate healing at a critically

short TG end. We now specifically point out that this finding supports a role for Tbf1 in the rescue of critically short telomeres that might arise, for example, due to fork arrest and breakage at the native TG_{1-3} ends.

(iii) As we pointed out in the original manuscript, Arneric et al. (2007) provide very strong evidence that Tbf1 sites in natural subtelomeric X elements (present at all telomeres) are involved in a pathway for telomerase activation at short telomeres.

(iv) Tbf1 has been shown to be required for telomere length homeostasis (Ungar et al. 2009. *Nucl Acids* Res **37**: 3840-9) based upon the finding that a hypomorphic DAmP allele has a short telomere phenotype. This result is consistent with a direct role for Tbf1 in telomerase regulation, though an indirect effect through its transcriptional activation function (Preti et al. 2010. *Mol Cell* **38**: 614-620) cannot be excluded. However, we have now discovered that the *tbf1*-D*i* mutant displays slight telomere elongation! (see **Fig. S8**). These results (one of which is our own novel finding) strongly argue for a direct (and complex) role of Tbf1 in length regulation of native telomeres in budding yeast.

(v) The role of Tbf1 in fission yeast telomere maintenance is controversial, and certainly not excluded. Although the referee is correct in pointing out that Cockell et al. (2009) do not favor a key role for SpTbf1 in telomere maintenance, their data and that of Pitt et al. (2008. *J. Biol. Chem.* **283**: 2693-701) clearly indicate a possible role for the protein in telomere length homeostasis. To the best of our knowledge, *in vivo* SpTbf1 binding genome-wide has not been examined, though the protein clearly binds with high affinity and specificity *in vitro* to sequences found at *S. pombe* telomeres. As in *S. cerevisiae*, the fact that SpTbf1 is required for viability (perhaps due to an essential transcriptional activation function) makes it difficult to determine possible telomeric roles by genetic methods. This may require the isolation and careful characterization of *tbf1-ts* alleles or separation-of-function alleles with telomere effect, both of which are beyond the scope of the present study.

We have now modified the text (principally at the end of the Discussion) to incorporate these new data and to more clearly emphasize the strong arguments in support of a direct physiological role for Tbf1 in telomere function of wild type cells. Again, we thank the referee for pointing out this weakness in our original manuscript, since this has significantly helped us to clarify this important issue in the revised manuscript.

Specific Points

1. The authors should show that loss-of-function in TBF1 results in telomere elongation or checkpoint activation in cells carrying endogenous sub-telomeres and telomeres. This would be one of key experiments to determine whether the proposed Tbf1 function is physiologically relevant.

As pointed out immediately above, this specific experiment is not feasible because the *TBF1* gene is essential for viability. However, a *TBF1* DAmP allele (presumably hypomorphic) displays telomere shortening (Ungar et al. 2009. *NAR* **37**: 3840-9) thus providing direct evidence for a role of the protein in telomere length homeostasis. Furthermore, as pointed out above, we have now found that the *tbf1*-D*i* mutant displays slight telomere elongation (**Fig. S8**). This situation is very reminiscent of Rap1, where some alleles (ts) exhibit telomere shortening whereas others display varying degrees of telomere elongation. We contend that these two results for *TBF1* mutants, taken together, strongly implicate Tbf1 as a direct regulator of telomere length in wild-type cells.

2. The authors argued that like Rap1, Tbf1 contributes specifically to telomere protection because other transcription factors (Bas1, Abf1, Reb1) did not protect DNA ends from degradation. The respective constructs contained binding site arrays of 9 or 24 tandem copies for each of these transcription factors. The 230 TAG repeat sequence may contain more than 30 copies of TTAGGG because TTAGGG is only 6 bp long. The authors need to show that Tbf1 effectively protects DNA ends if 24 TTAGGG repeats are placed nearby.

We in fact show that 10 T_2AG_3 repeats (60 bp) are sufficient to protect a DNA end from degradation (Fig. 1B), and to allow the end to be very efficiently healed (83% viability) by formation of a

telomere (see modified **Fig. S2**). This capability of the short T_2AG_3 array is impaired in both *tbf1*-D*i* and *vid22*-D *ygr071*-D mutants. Neither of the other arrays, some much longer, promotes any measurable protection in these assays.

3. Cdc13 binding protects telomeres from DNA degradation; that is, Cdc13 acts as a telomere cap. Cdc13 binds TTAGGG repeats as well as TG repeats (Alexander and Zakian, 2003 EMBO J, 1688-1696). The authors should examine whether Cdc13 binds the Bas1, Abf1 or Reb1 repeats after HO induction. If Cdc13 does not bind these repeats, the provided data do not support the idea that Tbf1 protects TTAGGG ends. The current data do not exclude the possibility that Cdc13 plays a key role in TTAGGG end protection.

The reviewer raises a very interesting point here. In fact, Cdc13 binds robustly not only to Bas1 and Reb1 repeats, but also to a DSB containing no repeats of any sort! (see **Fig. R5**). Oza et al. (2009. *Genes & Dev* **23**: 912-27) also demonstrated Cdc13 binding at irreparable DSBs. These data clearly show that Cdc13 binding per se is not sufficient to protect a DNA end, and thus clearly support our conclusion that Tbf1 plays a direct role in this function when targeted to an end through its DNA-binding site.

4. In Figure 1, Cdc13 binding data should be included. Because the authors argue that Cdc13 binding is critical for telomere addition, the tbf1-deletion-i mutation could affect Cdc13 binding.

We have measured Cdc13-myc binding in the *tbf1*-D*i* mutant and find, as expected, that it binds robustly to both short and long T_2AG_3 ends (see **Fig. R6**). Interestingly, its binding at the long ends appears to be stronger relative to the short, a situation opposite to that observed in *TBF1* wild-type strains. This is very likely a reflection of the (partial) loss of capping at the long ends in the *tbf1*-D*i* mutant, and the fact that they contain more TG repeat that could promote Cdc13 association. Whether this effect (increased relative Cdc13 binding) is related to elongation of the long tracts in the mutant background is still an unresolved issue.

5. In Figure 2, the authors need to confirm DSB induction by Southern blotting.
It is not clear whether HO expression indeed generates a DNA break into the cleavage site at the 230 bp TAG-CTA repeats. If the sequence is resistant to HO cleavage, it is not surprising that HO expression does not activate checkpoint.

This is of course a good point. We have performed the Southern blot and shown directly that both types of ends are efficiently cut (see **revised Fig. S2B**). We now point this out in the text. We have also shown that the distal marker (*LYS2*; see Fig. 1A), which is also present in the constructs in Fig. 2 (though not indicated for space reasons), is lost in most cells following galactose induction. This is also a direct indication that cutting is efficient.

6. Does the tbf1-deletion-i mutation affect the expression level of Est1 or Est2 in Figure 3? It is curious that Est1 or Est2 associates with DSBs poorly in tbf1-Di cells.

Neither Est1 nor Est2 protein levels are reduced (or increased) in *tbf1*-D*i* cells (see **Fig. R7**), so we do not think that this is the reason for apparently weaker association of these proteins at breaks. We have noted weaker association of essentially every protein we have looked at in *tbf1*-D*i* cells, compared to WT, which might be related to the fact that the mutant cells have a growth defect. We thus think that a comparison of the magnitude of fold enrichment between *tbf1*-D*i* cells and WT is problematic, and have tried to restrict ourselves to comparisons within the same strain background at different types of breaks.

7. Southern blots, which monitor telomere addition, should be included in Figure 6. There is so far no evidence that Rap1 binding is correlated to telomere addition.

As we explain above, there is indeed published evidence that Rap1 binding, as measured by ChIP, is correlated to telomere addition at DSBs. We also include a Southern blot to confirm this (see **Fig. S5**) and mention this in the text.

8. Figure 7. Viability in wt and mec1 deleted cells after HO induction should be included to support telomere addition at DSB ends in mec1 deletion mutants. The authors need to confirm telomere

addition by Southern blotting.

We have now done the survival experiment suggested by the referee (excellent point), comparing *mec1*-D *sml1*-D *rad52*-D cells to *sml1*-D *rad52*-D control cells. Our measure of survival through telomere formation is to count Ade^+ Lys^-, where HO cutting has presumably occurred (loss of distal *LYS2* gene) and telomere formation has left the *ADE2* gene (immediately upstream of the HO site) intact. Strikingly, and as predicted by our ChIP data, survival is higher (by about 2-fold) in the absence of Mec1 (see **Fig. S7**). We note that Zhang and Durocher have obtained very similar results, but in their case in a *pif1* mutant background (where overall survival is much higher due to the absence of the Pif1 telomerase inhibition pathway). These new data very strongly support the conclusions from the original ChIP experiments, namely that Mec1 inhibits Cdc13 association at a DSB and subsequent telomerase action there.

We have also performed Southern blots as the reviewer has requested, but are unable to detect evidence for elongation of the DSB using this method. The Southern blot assay is apparently not sensitive enough to detect what is most probably a low frequency of elongation events. This conclusion is strongly supported by data in Zhang and Durocher (2010. *Genes & Dev*; confer their Fig. 4) who do a similar experiment, but in a *pif1* mutant background, where telomere healing rates are actually much higher than in our *PIF1* cells. Perfectly consistent with our conclusions, their data show that *mec1* mutation increases telomere healing at DSB ends (called TG_0 in their paper), by about 2-fold. As pointed out above, the proxy that we use in these experiments to detect telomerase elongation is Rap1 ChIP, which has been verified in two previous publications. This assay is extremely sensitive: please note that Rap1 fold-enrichment at a TG80 tract before cutting (Fig. S1) is roughly 40-fold higher than that detected at the DSB in *mec1* cells. I would also emphasize that we have now confirmed, as detailed above, that the increased Rap1 binding at the DSB in *mec1* cells is a statistically significant result. In addition, we report a novel finding highly relevant to mechanism (Fig. 7C), namely that Cdc13 binding also increases at the DSB in *mec1* cells. We point out in passing that this novel finding is also consistent with telomerase addition, though not a proof.

9. There is no figure legend for Fig. S5. It is impossible to evaluate the data.

We thank the reviewer for pointing out this omission, which has now been corrected.

10. Telomeres of fission yeast consist of long TTAGGG repeats. The fission yeast Tbf1 protein binds telomeric repeat TTAGGG but does not appear to protect telomere ends from checkpoint activation or play a key role in telomere maintenance. The authors need to discuss the difference between budding and fission yeast Tbf1 functions in "Conclusions and implications for telomere evolution".

The reviewer raises an interesting but complex issue here. As we pointed out above, SpTbf1 function is still poorly understood and indeed a controversial point. While it is clearly true that Rap1 in budding yeast and Taz1 in fission yeast are the key duplex telomere-repeat binding proteins for capping and telomere length regulation, back-up roles for Tbf1 in both organisms are not at all excluded. Indeed our results, as well as those of others, strongly support such a role for Tbf1 in budding yeast (again as detailed above). We have added to our Discussion a reference to the possibility that SpTbf1 plays a similar role, confirmation of which would clearly require considerable additional investigation.

Minor points

1. The detailed culture condition should be included in the figure legend, because the 230 bp TAG sequence became shorter in Fig. 4 and Fig. 5 but did not in Fig. 1.

The experiments in Fig. 1 were performed in wild type cells, those in Fig. 4 in *mre11* and Fig. 5 *tel1* mutants. This is why the telomeres shorten in the latter two cases.

2. Fig, S5 (page 13, line 5) should be Fig, S4.

We thank the reviewer for pointing out this error, which has now been corrected.

Fig. R1 (Referee #1 : question 1). Quantification of Cdc13 and Est1 recruitment using % input or fold enrichment.

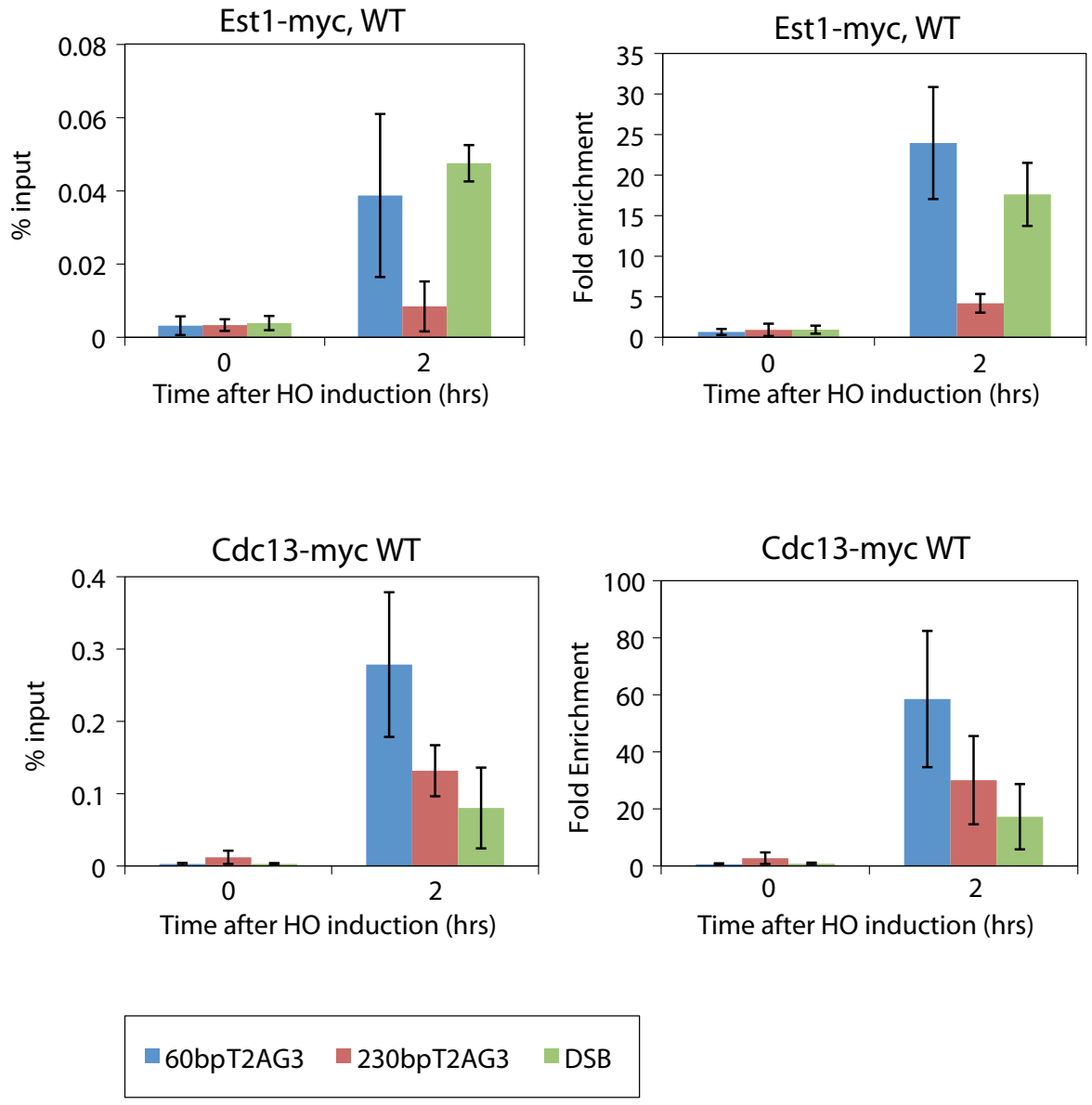
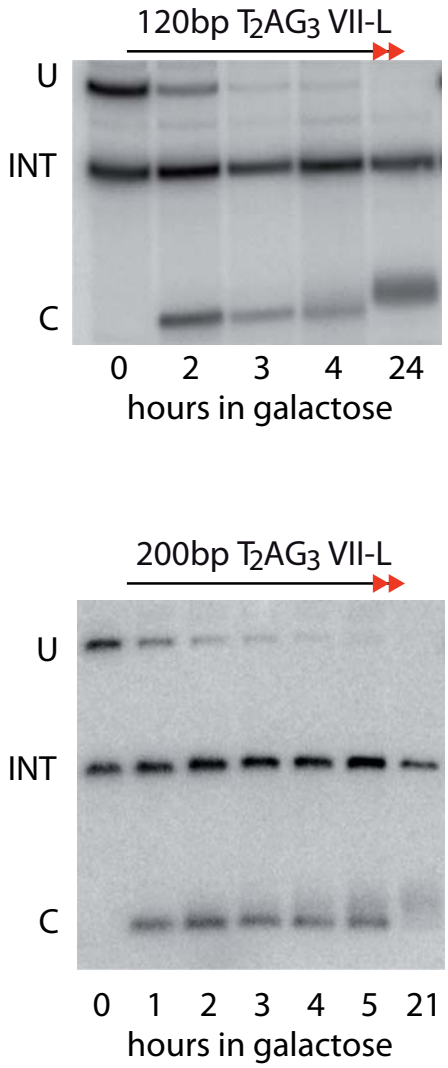


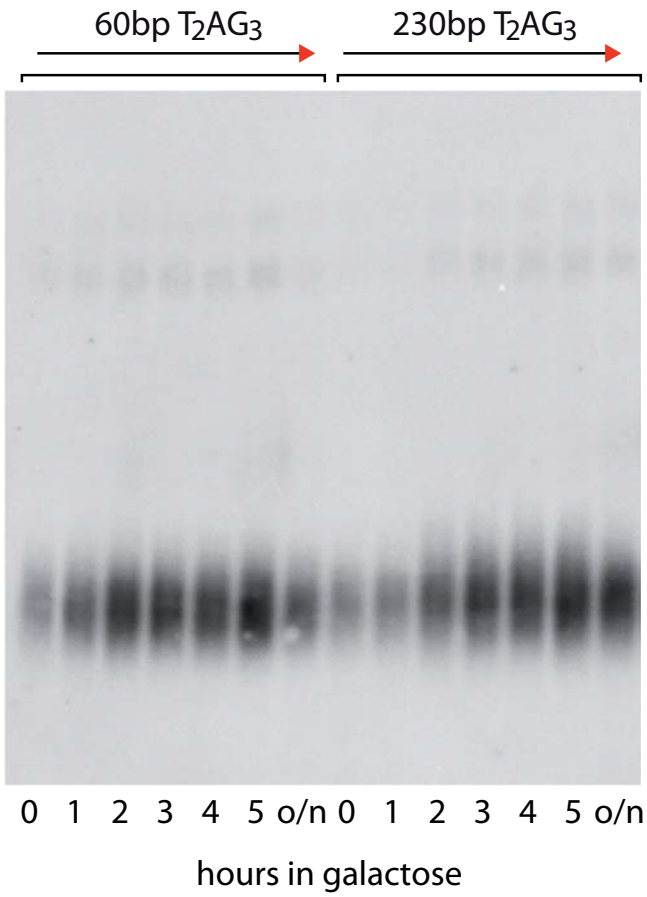
Fig. R2 (Referee #2 : question 1)



Southern blots monitoring behaviour of 120bp and 200bp T2AG3 after cut. An internal loading control ("INT"), a fragment arising before HO cutting ("U"), and a fragment derived from "U" following HO digestion ("C") are marked.

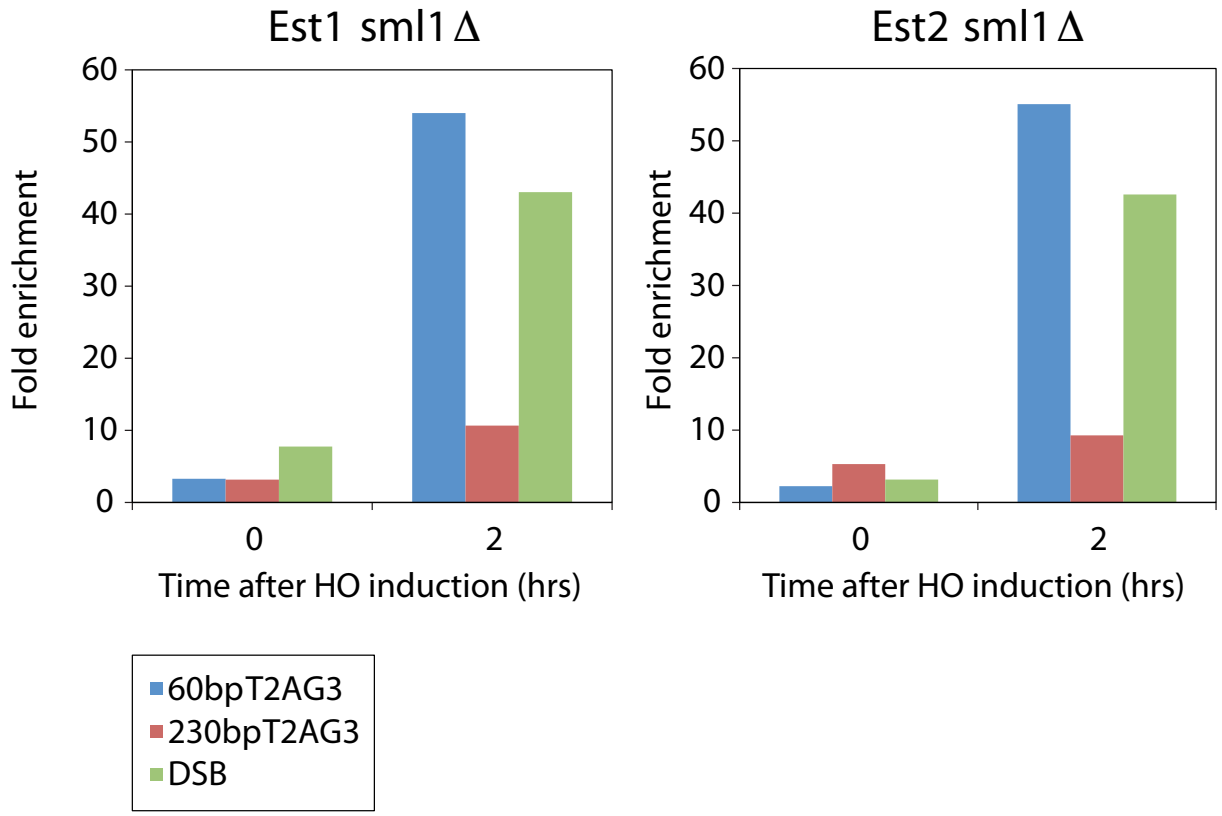
Fig. R3 (Referee #2 : question 5)

Endogenous telomeres are not modified following HO induction



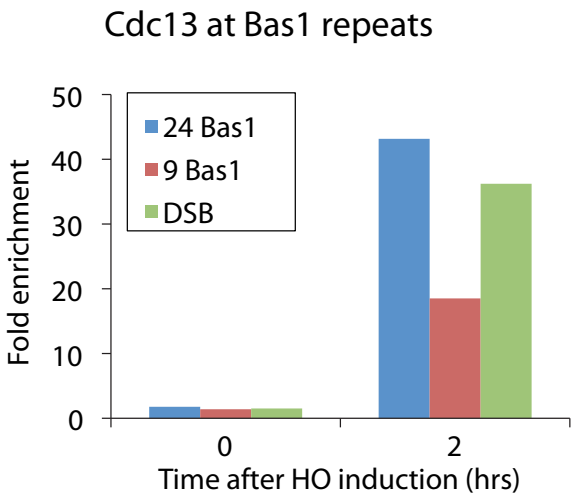
Southern blot monitoring behavior of endogenous Y' telomeres after cut in strains harboring 60 bp or 230 bp T2AG3 constructs.

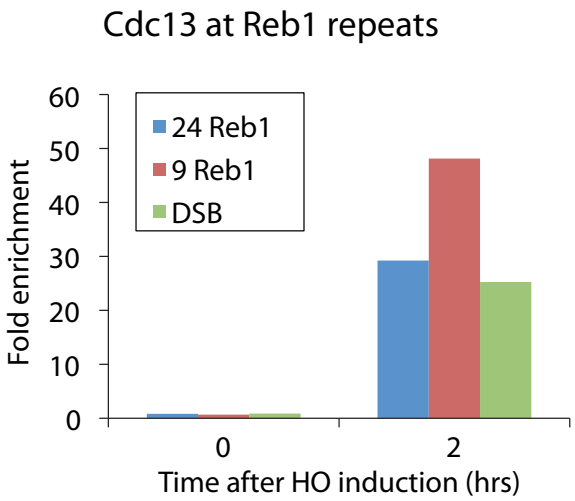
Fig. R4 (Referee 2 : minor comment 4). Telomerase recruitment in sml1Δ



Analysis by ChIP of Est1 and Est2 binding in sml1Δ mutant

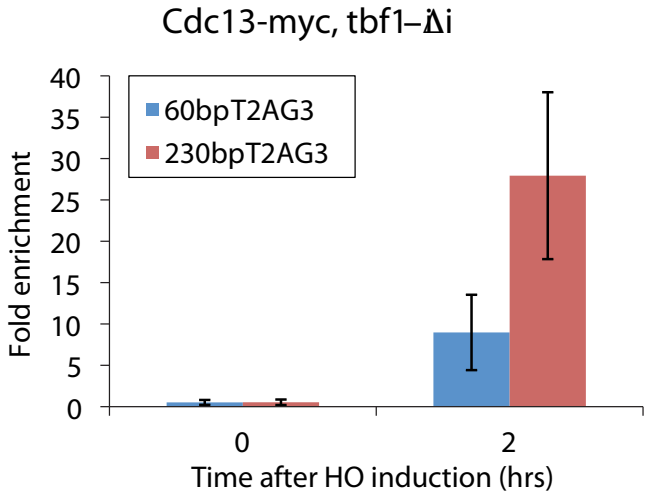
Fig. R5 (Referee #3 : question 3) Cdc13 binding at Bas1 and Reb1 arrays





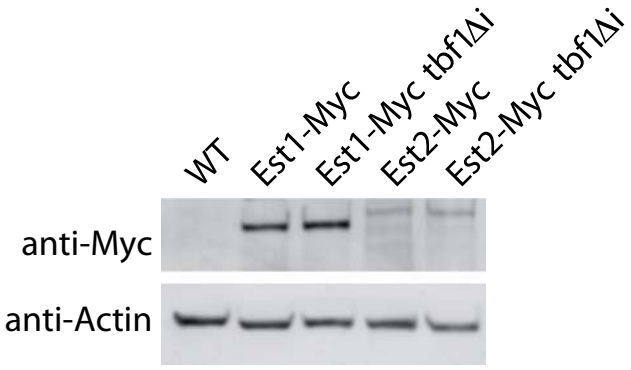
Analysis by ChIP of Cdc13 binding at Bas1 and Reb1 repeats

Fig. R6 (Referee #3 : question 4) Effect of tbf1-Δi on Cdc13 binding



Analysis by ChIP of Cdc13 binding at 60 bp and 230bp T2AG3 in tbf1Δi mutant

Fig. R7 (Referee #3 : question 6) Est1 and Est2 expression in tbf1-Δi



Analysis by Western-blot of Est1 and Est2 protein levels in WT and tbf1Δi mutant. Antibody against Myc-epitope was used to detect Myc-tagged Est1 and Est2 and antibody against actin was used as loading control.

2nd Editorial Decision 04 August 2011

Thank you for submitting your extensively revised Tbf1 manuscript for our consideration. The three original referees have now looked at it once more, and I am pleased to inform you that all of them consider the manuscript substantially improved and now in principle suitable for publication in The EMBO Journal. As you will see, only referee 3 retains some minor reservations (the significance of which I additionally discussed with one of the other referees). Before formal acceptance, I would like to ask you to fix the first point (better descriptions of a particular set of experiments), while point 2 in my opinion has been well addressed and discussed already. Regarding the third point on the Mec1 data: here referee 3 still remains unconvinced by the new data and responses, and proposes to leave this part out. I realize that these data may be a bit more peripheral to the main Tbf1 story, so I would leave it up to you to decide whether to retain or to remove this part. Should you decide to retain it, I would however recommend that you either strengthen these data according to the referee's criticism, or tone them down to reflect the remaining caveats.

Three additional editorial points that will need to be addressed with the final version:
- please add a 'conflict of interest' statement to the article text
- please upload each of the MAIN figures as a single file - we need this for production
- finally, we noticed on our routine pre-acceptance CrossCheck procedures that extensive parts of the Materials & Methods sections represent nearly verbatim copies of passages from the M&M section of your 2007 G&D paper (Negrini et al): in order to avoid possible allegations of self-plagiarism at a later stage, I hope you appreciate that these passages would need to be slightly rephrased/modified before publication.

I am therefore returning the manuscript to you for a final round of minor revision. Please, get the modified manuscript back to us as soon as possible; following resubmission we should then be able to swiftly proceed with acceptance and publication of the paper!

Sincerely yours,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

Overall I think the authors have gone very far in this paper and the revisions are thorough. In my opinion the paper is very good and appropriate for EMBO journal.

Referee #2

Overall, this paper is significantly improved through rewriting and through the addition of new data and new calculations. Importantly, the authors have alleviated all my concerns. I am much more enthusiastic that I was initially.

Referee #3

In the original manuscript, the authors provided several data suggesting that Tbf1 protects DNA ends from DNA degradation and down-regulates telomere extension and checkpoint activation. I raised three major questions to be addressed before publication.
Although the authors showed several data, they addressed my questions imprecisely or provided data lacking detailed information.

The authors have uncovered the mechanism of how Tbf1 negatively regulates telomere length. This is a significant contribution to telomere biology. The authors should focus on key findings by deleting irrelevant issues.

1. Tbf1 contributes to telomere protection. However, the authors show that Tbf1 acts so only on a 230 bp TTAGGG array. Is the proposed Tbf1 function physiologically relevant? (Specific Point 1).

I found that the authors argued appropriately why Tbf1 contributes to telomere protection in Discussion of the revised version, and the proposed idea is appealing. I consider, however, that the newly added data are somewhat misleading.

The authors provided that the tbf1-di mutant displays telomere addition after restreaking (Fig. S8). It is difficult to evaluate the data because the authors did not provided detailed experiment procedures. The figure legend does not describe how cells were grown before and after being streaked.

The 11 bp TG11-60 bp T2AG3 experiment (Fig. S9) experiment does not make stronger argument because the 60 bp T2AG3 sequence alone stimulates telomere addition (Fig. 1, 4 and 5).

2. Tbf1 contributes specifically to telomere protection while related transcription factors (Bas1, Abf1, Reb1) does not.
There is rich evidence showing that Cdc13, well-known as a telomere protecting protein, binds to single-stranded Tbf1 binding sequence. However, there is no report showing that the binding sequence for Bas1, Abf1 or Reb1 is recognized by Cdc13. It is possible that the Bas1, Abf1 or Reb1 binding sequence fails to prevent DNA deradation because these sequences are not covered with Cdc13? (Specific Point 3).

The data in Fig. R5, together with Fig. 7, show that Cdc13 binding at the Tbf1 binding sequence is much more robust than that at those for Bas1, Abf1 and Reb1 binding sequence. DNA ends containing Bas1, Abf1 or Reb1 binding sites are degraded. Since DSB ends are very quickly degraded and converted to ssDNA tracts, Cdc13 could be less densely positioned at DNA ends containing Bas1, Abf1 or Reb1 binding sites than at those containing Tbf1 binding sites. Cdc13 may bind at a remote region but not near the Bas1, Abf1 or Reb1 end. It thus remains possible that Cdc13 plays a major role in telomere protection. I suggest that the authors delete or weaken the point that Tbf1 acts as a telomere cap.

3. Mec1 inhibits telomere addition by inhibiting Cdc13 binding. Telomere addition heals DSB ends. To support the model, I asked the authors to show that telomere addition and cell viability after DSB induction is increased in mec1 mutants (Specific Point 8).
The authors did not show good evidence of telomere addition in mec1 mutants.
The interpretation of the survival assay would be misleading. Only 1% of cells were survived for both MEC1 and mec1 mutant cells. There is no statistic significance. Telomere addition, estimated by monitoring the loss of a selection marker, after overnight DSB induction is 15% and 31% for MEC1 and mec1 mutant cells, respectively. Overall, these data suggest that most cells did not repair DSBs by telomere addition. The Mec1 function is not so relevant to the Tbf1 story. I suggest that the authors remove the Mec1 function section and concentrate on more important findings.

2nd Revision - authors' response 31 August 2011

We would like to thank you again, as well as the referees, for your time and effort in evaluating our manuscript. We are of course delighted that you have decided to accept the manuscript, in principle, pending a proper response to the 3rd point of referee #3, the provision of information regarding this referee's 1st point, and correction of editorial issues.

Regarding the 1st point of referee #3, we now provide a detailed description of how the cells were grown prior to telomere length analysis. This is a standard assay in the field, but the referee is correct to point out that we should have more explicitly stated exactly how this experiment was

performed. Regarding his/her comment on Fig. S9 ("…experiment does not make stronger argument because the 60 bp T2AG3 sequence alone stimulates telomere addition), we actually disagree, since previous experiments had shown only that external T_2AG_3 sequences could stimulate telomere addition. Our new experiment shows that a more internal T_2AG_3 array, similar to what is found at native telomeres, can rescue a critically short TG-repeat end. This is indeed another piece of evidence for a Tbf1 capping function with physiological relevance.

As you state in your letter, the 2nd point raised by this referee has already been discussed and addressed. I would simply add here that we do not rule out a role for Cdc13 in capping at T_2AG_3 arrays, and specifically state in the text that the Tbf1 (and Rap1) capping functions act in parallel to that of Cdc13 (pg. 15). The important point here is that our findings with the *tbf1*-D*i* mutant (e.g. Fig. S2) clearly demonstrate a direct role for this protein in capping. Furthermore, long Tbf1 arrays bind less Cdc13 than short arrays yet are more effectively capped. Therefore, there is simply no way to escape the conclusion that Tbf1 plays a role in capping at T_2AG_3 array ends, irrespective of any role that Cdc13 might play. Regarding the magnitude of Cdc13 binding at Bas1, Abf1 and Reb1 array ends relative to Tbf1 site ends, we consider the differences to be rather small and very unlikely to explain the enormous difference in end stability that we observe. Additional experiments, beyond the scope of the present study, would be required to define the role of Cdc13 in capping at these ends.

Regarding the 3rd point, we worry that the referee #3 has not completely understood this admittedly complex experiment. This experiment is similar to an extensive set of experiments reported recently by Zhang and Durocher (*Genes Dev*. 2010 **24**: 502-515) that evaluated telomere formation at a set of short TG ends in different genetic backgrounds (including *mec1*-D). In almost all of the Zhang & Durocher experiments the cells carried at *pif1-m2* mutation, which significantly increases telomere formation compared to *PIF1*, the genetic background we used (see their Fig. 3 for the comparison). The fact that "only 1% of cells were (sp) survived for both *MEC1* and *mec1* mutant cells" is immaterial. What is important is that the survival frequency was 2-fold higher in the *mec1* mutant cells compared to wild type. This result is statistically significant, as we pointed out in the legend to Fig. S7: "Survival frequency is statistically different ($p<0.001$, chi-square test) in *mec1*-D compared to control." As we also pointed out in our previous letter, this is nearly exactly what Zhang & Durocher found, though in a different experimental set-up where telomere formation overall was significantly increased by the presence of the *pif1-m2* mutation. This result (Fig. S7) clearly supports our conclusions from the ChIP data (Fig. 7) showing that the *mec1* mutant cells display increased Rap1 at the DSB relative to wild type (evidence for telomerase-mediated elongation) as well as increased Cdc13 binding. We infer from this that in wild type cells Mec1 acts to prevent telomere formation at accidental DSBs, an event that would normally be lethal (but in our experiment promotes survival, since the break is near a chromosome end, beyond any essential gene). Parenthetically, the reviewer's claim that "these data suggest that most cells did not repair DSBs by telomere addition" may not even be correct, since we do not know what fraction of the cells actually experienced an HO cut.

While we agree with the referee that these findings are not so obviously relevant to the Tbf1 story, they do address an important point regarding telomere formation at DSBs, which is certainly a highly related subject. Furthermore, this question has been the subject of two very recent high-profile papers (Zhang & Durocher *Genes & Dev.*; Makovets & Blackburn *Nature Cell Biology*). Since our data provide new molecular insights into this issue (and the first demonstration that Mec1 has an effect on telomere formation in cells carrying wildtype Pif1), we would like to include them in the present manuscript since we are confident that they will be of high interest to researchers in the field.

I hope that you find the above, as well as modifications to the text, a suitable response to the most recent critiques of referee 3, and that you can thus approve the revised manuscript for publication.

Once again, thanks for all of your time and effort (and to the referees), which we believe has resulted in a significantly improved paper.

3rd Editorial Decision 01 September 2011

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the remaining points, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

