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HOT1 is a mammalian direct telomere repeat binding protein contributing to telomerase recruitment

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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 21 December 2012

Thank you for submitting your manuscript on HOT1 as a telomere repeat binding protein for consideration by The EMBO Journal. We have now received feedback from three trusted expert referees who had agreed to evaluate your study. I am pleased to inform you that all of them consider your findings interesting and potentially important for the field. However, they also raise a number of concerns that would need to be satisfactorily clarified before publication. As you will see from the reports of referees 1 and 3 (copied below), they both bring up several well-taken experimental and presentational issues to be addressed. Referee 2 was unfortunately not able to provide a full, formal report, but indicated that his/her only main concern is the missing demonstration of in vivo telomere binding dependence on the HOT1 homeodomain.

In conclusion, we should be happy to consider a revised version of the manuscript further for publication, should you be able to adequately address the following key points:

-The homeodomain dependence of HOT1 in vivo telomere binding should be confirmed as requested by referee 2 (see above), by studying telomere localization (e.g. with immuno-FISH) of HOT1 lacking the homeodomain or (even better) with your structure-based mutations that abrogate DNA binding

- The proposed role in TERT recruitment should be strengthened as requested in the main experimental points of referees 1 and 3, by assaying telomerase chromatin binding or telomere ChIP

in cells with HOT1 gain (overexpression) and loss (knockdown, or possibly testing dominant-negative effects of DNA-binding deficient HOT1) of function. Also, some answers to referee 3's questions A-D would be certainly improve the manuscript.

- From an editorial point of view, it will be essential to considerably expand both the introduction and the discussion (each of them currently barely exceeding one manuscript page!) to provide a more scholarly overview over the relevant background and to better contextualize the results. In this respect, the previous identification of HOT1/HMBOX1 telomere association by Dejardin and Kingston has to be mentioned upfront already in the introduction, as well as the earlier findings on HMBOX1 as a DNA-binding transcriptional repressor protein (Chen et al 2006). Consequently, also the headline of the first result section paragraph should not claim 'Hot1 is a novel direct telomere repeat binding protein' but more accurately read 'Identification of Hot1 as a direct telomere repeat binding protein' - I do not feel these changes would distract from the interest of your present work!

- In a similar vein, I feel a more adequate and explicit title would be appropriate, possibly also incorporating the telomerase recruitment aspect if substantiated by revision experiments. My suggestion would be something along the lines of "HOT1 is a vertebrate telomere repeat binding protein with roles in telomerase recruitment".

- Finally, the structural analyses need to be better described and contextualized. Firstly, summary tables of data collection and refinement statistics, as well as PDB deposition and provision of the respective PDB accession codes is an essential prerequisite for acceptance. However, I also notice that the Method section on page 19 makes mention of a previous HMBOX1 homeodomain NMR structure (for which the wrong PDB code seems to be given) used here as a search model - this previous structure determination again has to be mentioned in the results/discussion section; again it would not appear to distract from the novelty of the HOT1-DNA co-crystal structure described here!

I should remind you that it is our policy to consider only one single round of major revision, and that it is therefore essential to satisfactorily address all the main points at this stage. Therefore, please directly respond to my above points in your response letter with the revised version, to make clear how you addressed each of these issues. Also please make sure to adequately respond to the various other more specific points raised by reviewers 1 and 3. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Kappei et al. reports the characterization of HOT1, a novel factor that binds telomeric DNA with sequence specificity, similarly to TRF1 and TRF2. Although HOT1 was previously identified as a component of the telomeric complex using PICh (proteomics of isolated chromatin segments; Dejardin and Kingston, 2008), the current study clearly establishes HOT1 as a telomeric DNA-binding and telomerase-interacting factor, essential for telomere length maintenance in mammalian cells. As such, the paper is breaking new ground and is suitable for publication in EMBO J.

The authors demonstrate that HOT1 binds telomeres in vitro and in ChIP assays and that it localizes to telomeres in ES cells and spermatocytes. The co-crystal structure of the HOT1 DNA binding domain and telomeric repeats is reported in comparison to that of TRF1. This is the first HOT1 crystal structure, as far as I am aware, and provides substantial detail into its mode of binding to the telomeric DNA substrate.

HOT1 localizes to a subset of telomeres in human cells and is required for telomere elongation. This suggests the possibility that the HOT1 subset contains telomeres actively elongated by telomerase. Whilst such a hypothesis cannot be rigorously tested in human cells, the authors demonstrate that HOT1 interacts with active telomerase components in co-immunoprecipitation assays and that it associates with Cajal bodies, the sites of telomerase biogenesis, in immuno-localization experiments. Thus, the most likely function of HOT1 is to target telomerase to elongating telomeres. To strengthen such a role, TERT binding to chromatin in cell fractionation or directly to telomeres in ChIP assays should be tested using HOT1-deficient cells. In my experience, a clean gene knock-out should be used in these approaches, as RNAi depletion does not usually abolish completely protein expression and residual protein may be sufficient to target TERT to telomeres. Even if such experiments will have to await availability of a HOT1 KO mouse model, the current study represents a fascinating entry point into the role of HOT1 in telomere stability and its functional interactions with telomerase.

Minor comments:

-p3, paragraph 2 - Third sentence should end at Zhong et al 2012.
-p6, paragraph 1 - Sentence "Exhibiting similar..." should be re-formulated.
-p6, paragraph 2 - "To further understand..." rather than "To further manifest..."
-p7 - residue K335 mutation to Ala seems to switch sequence specificity to non-telomeric DNA binding; is it possible that this residue is essential for the specificity to telomere binding?
-p9 - one possible explanation for the observed lack of HOT1 association with all telomeres is that some HOT1 foci localize to very short telomeres which are below the limit of detection by FISH or TRF1 immunofluorescence. Telomeric DNA may be completely eroded in these telomeres, at the same time these may be the telomeres marked for elongation by telomerase.
-Fig. 3b: helix 3 referred to in p8 should be labelled as in Fig. 2c
-Discussion should mention that HOT1 was previously identified (Dejardin and Kingston, 2008) and how the current study furthers the understanding of its function at telomere.

Referee #3 (Remarks to the Author):

The authors have determined that the previously identified HOT1 protein is a double stranded telomere binding protein that is involved in telomere length regulation via regulating access of telomerase to the telomeres during telomere elongation. The data presented in this manuscript provides novel insights into the mechanism of telomere length regulation by telomerase; it raises important questions regarding regulation of telomerase access to the telomeres and enriches the telomere field significantly. There are, however, several issues listed below that need to be addressed before accepting the manuscript for publication in EMBO.

MAJOR ISSUES
Title: Change to "HOT1, a novel vertebrate telomere binding protein recruits telomerase to telomeres"

CST complex dependent telomere maintenance in vertebrates needs to be discussed in the manuscript by the authors. They refer to the shelterin complex as if this is the only complex involved in telomere maintenance

Page 6, first paragraph: "HOT1 contains a homeobox domain (Chen et al, 2006), suggesting that it may bind DNA directly." This protein was first identified by Chen et al and the authors showed that HMBOX1 is a transcription repressor. The authors need to modify their abstract and/or Introduction

sections of the manuscript accordingly

Tables: Table of x-ray crystal data collection and refinements statistics is missing

Discussion section, page 14, pargarph 2: "The putative mechanism may involve recruiting telomerase-containing Cajal bodies to telomeres, and thus promoting telomerase association with telomeres, perhaps through additional interactions involving TPP1".
This is an important aspect of this work as it has been well established that TPP1 of the shelterin complex recruits telomerase to the telomeres. The research findings presented here raise several important questions: A) Does HOT1 act alone in recruiting telomerase to the telomeres and if so, is this cell cycle dependent? B) Is it possible that HOT1 and TPP1 together coordinate telomerase recruitment to the telomeres and why other screens haven't picked up this interaction previously? C) Is HOT1 an integral component of the Shelterin complex? D) Is there a direct contact between TPP1 and HOT1?

MINOR ISSUES
Abstract first sentence: "Telomeres are repetitive DNA structures that together with the shelterin complex protect the ends of chromosomes." There is now evidence, which supports telomere protection by the shelterin and CST complex in vertebrates

Title and Abstract: Authors need to specify which organism or species are they referring too early on. Reason: Cdc13 recruits telomerase to the telomeres in yeast and TPP1 in vertebrates

End of Introduction section: Re-write sentence: "In addition, we show that HOT1 associates with the active telomerase complex and characterize HOT1 to be localized to telomeres in settings of active processing."

Results section, page 6, first paragraph: "To determine whether HOT1 was detected in our assay because of a strong association with the shelterin complex or direct binding to TTAGGG repeats, we performed DNA binding assays with HOT1 in vitro."
The authors do not discuss shelterin association of HOT1 in this section of the results so it needs to either be taken out or the appropriate data shown and discussed.

Results section, page 6, last paragraph: "The three longer constructs Q144-A345, L156-A345 and G233-A345 all bound to immobilized telomeric dsDNA baits .... "
What is the binding affinity of HOT1 for double stranded telomeric DNA?

Figure 2a. What concentration of DNA probes and protein were used?

Figure 2C: Authors should label the protein and nucleic acid residues in Figure 2C

1st Revision - authors' response 09 April 2013

Thank you very much for giving us the opportunity to submit a revised version of our manuscript. Your comments and the suggestions from the referees have been very helpful to strengthen our conclusions. As you will see in the revised manuscript and in the rebuttal letter we have been able to address all the key points, including the homeodomain dependent in vivo telomere binding, a contextualized structural analysis, an expansion of the introduction and discussion and a more adequate and explicit title. Finally, we now demonstrate a role of HOT1 in TERT recruitment utilizing Hot1 knockout MEFs.
We are confident that with these changes our manuscript will prove suitable for publication in the EMBO Journal and we are looking forward to your feedback at your earliest convenience.

In conclusion, we should be happy to consider a revised version of the manuscript further for publication, should you be able to adequately address the following key points:

-The homeodomain dependence of HOT1 in vivo telomere binding should be confirmed as requested by referee 2 (see above), by studying telomere localization (e.g. with immuno-FISH) of HOT1 lacking the homeodomain or (even better) with your structure-based mutations that abrogate DNA binding

To address this point we expressed FLAG-HOT1 wt, FLAG-HOT1ΔhomeodomainandFLAG-HOT1 R339A in mouse embryonic stem cells. FLAG-HOT1 R339A represents one of our structure-based mutations that abrogate DNA binding (Fig. 2e). Similar to our co-localization data with endogenous HOT1 (Fig. 4), FLAG-HOT1 wt shows a punctuated nuclear pattern and localizes to several TRF1 foci. Both the homeodomain deletion and the R339A point mutant do not localize to telomeres (new Supplementary Fig. S4b). This data confirms that the homeodomain is indeed essential for *in vivo* binding of HOT1 to telomeres.

- The proposed role in TERT recruitment should be strengthened as requested in the main experimental points of referees 1 and 3, by assaying telomerase chromatin binding or telomere ChIP in cells with HOT1 gain (overexpression) and loss (knockdown, or possibly testing dominant-negative effects of DNA-binding deficient HOT1) of function. Also, some answers to referee 3's questions A-D would be certainly improve the manuscript.

We have now established a *Hot1* genetrap mouse line and generated mouse embryonic fibroblasts (MEFs) to address TERT recruitment in a clean loss-of-function background (as suggested by referee 1). Based on the insertion of the genetrap construct in the first intron of the *Hot1* gene and the start codon residing in exon2, *Hot1*$^{Gt(pU-21T)346Card/(Gt(pU-21T)346Card}$ mice are functionally a knock-out. We confirmed complete loss of HOT1 protein by immunofluorescence stainings (new Fig. 4c) and therefore refer to the genetic background of these MEFs as *Hot1*$^{-/-}$. Using *Hot1*$^{+/+}$ and *Hot1*$^{-/-}$ MEFs derived from littermate embryos, we assayed telomerase binding to the chromatin fraction. To this end, we used the experimental settings as previously described by Tejera et al. (Tejera et al., Dev Cell, 2010) for the analysis of TPP1 as a telomerase recruitment factor. An aliquot of the TERT antibody and *Tert*$^{-/-}$ MEFs (serving as a positive control) used in the Tejera et al. study were kindly provided to us. Under these experimental conditions we were able to detect a TERT band in the chromatin fraction that was absent in extracts from *Tert*$^{-/-}$ MEFs. This band was equally depleted in *Hot1*$^{-/-}$ samples (new Fig. 8d), reminiscent of the results on *Tpp1*$^{\Delta/\Delta}$ MEFs by Tejera et al. Thus, HOT1 is important for telomerase chromatin binding, strongly supporting the idea that HOT1 indeed acts as a telomerase recruitment factor.
We have majorly answered questions A-D, also including additional immunoprecipitation experiments (new Supplementary Fig. S6b and S6c), and incorporated this into the discussion section (please see below for details).

- From an editorial point of view, it will be essential to considerably expand both the introduction and the discussion (each of them currently barely exceeding one manuscript page!) to provide a more scholarly overview over the relevant background and to better contextualize the results. In this respect, the previous identification of HOT1/HMBOX1 telomere association by Dejardin and Kingston has to be mentioned upfront already in the introduction, as well as the earlier findings on HMBOX1 as a DNA-binding

transcriptional repressor protein (Chen et al 2006). Consequently, also the headline of the first result section paragraph should not claim 'Hot1 is a novel direct telomere repeat binding protein' but more accurately read 'Identification of Hot1 as a direct telomere repeat binding protein' - I do not feel these changes would distract from the interest of your present work!

We have expanded both the introduction and discussion and contextualized more clearly the referred work of Dejardin and Kingston as well as the description of HMBOX1 as a putative transcriptional repressor. The headline of the first result section was changed to "Identification of HOT1 as a direct telomere repeat binding protein".

- In a similar vein, I feel a more adequate and explicit title would be appropriate, possibly also incorporating the telomerase recruitment aspect if substantiated by revision experiments. My suggestion would be something along the lines of "HOT1 is a vertebrate telomere repeat binding protein with roles in telomerase recruitment".

We appreciate the suggestion and have revised the title to "HOT1 is a mammalian direct telomere repeat binding protein contributing to telomerase recruitment".
As we exclusively provide experimental data from mammalian sources (human and mouse) we prefer to limit the claim to mammals at present. Certainly, the HOT1 gene is conserved in the entire vertebrate lineage and there are homologs in ascidians, echinodermata and nematodes, but functional conservation remains to be addressed experimentally in the future.

- Finally, the structural analyses need to be better described and contextualized. Firstly, summary tables of data collection and refinement statistics, as well as PDB deposition and provision of the respective PDB accession codes is an essential prerequisite for acceptance. However, I also notice that the Method section on page 19 makes mention of a previous HMBOX1 homeodomain NMR structure (for which the wrong PDB code seems to be given) used here as a search model - this previous structure determination again has to be mentioned in the results/discussion section; again it would not appear to distract from the novelty of the HOT1-DNA co-crystal structure described here!

We now provide crystallographic statistics in Table 1 and the structure is deposited under the PDB code 4J19. The PDB code for the homeodomain NMR structure has been corrected to 2CUF and is emphasized in the results section.

Referee #1 (Remarks to the Author):

The manuscript by Kappei et al. reports the characterization of HOT1, a novel factor that binds telomeric DNA with sequence specificity, similarly to TRF1 and TRF2. Although HOT1 was previously identified as a component of the telomeric complex using PICh (proteomics of isolated chromatin segments; Dejardin and Kingston, 2008), the current study clearly establishes HOT1 as a telomeric DNA-binding and telomerase-interacting factor, essential for telomere length maintenance in mammalian cells. As such, the paper is breaking new ground and is suitable for publication in EMBO J.

The authors demonstrate that HOT1 binds telomeres in vitro and in ChIP assays and that it localizes to telomeres in ES cells and spermatocytes. The co-crystal structure of the HOT1 DNA binding domain and telomeric repeats is reported in comparison to that of TRF1. This is the first HOT1 crystal structure, as far as I am aware, and provides substantial detail into its mode of binding to the telomeric DNA substrate.

HOT1 localizes to a subset of telomeres in human cells and is required for telomere elongation. This suggests the possibility that the HOT1 subset contains telomeres actively elongated by telomerase. Whilst such a hypothesis cannot be rigorously tested in human cells, the authors demonstrate that HOT1 interacts with active telomerase components in co-immunoprecipitation assays and that it associates with Cajal bodies, the sites of telomerase biogenesis, in immuno-localization experiments. Thus, the most likely function of HOT1 is to target telomerase to elongating telomeres. To strengthen such a role, TERT binding to chromatin in cell fractionation or directly to telomeres in ChIP assays should be tested using HOT1-deficient cells. In my experience, a clean gene knock-out should be used in these approaches, as RNAi depletion does not usually abolish completely protein expression and residual protein may be sufficient to target TERT to telomeres. Even if such experiments will have to await availability of a HOT1 KO mouse model, the current study represents a fascinating entry point into the role of HOT1 in telomere stability and its functional interactions with telomerase.

Based on the suggestion by this reviewer we tested TERT binding to chromatin by cell fractionation in a *Hot1*$^{-/-}$ background. As expected for a role in telomerase recruitment the TERT signal was depleted from the chromatin fraction (for details please see above).

Minor comments:

-p3, paragraph 2 - Third sentence should end at Zhong et al 2012.

We have shortened this specific sentence accordingly and it now reads: "Furthermore, TPP1 has been shown to be required for recruitment of telomerase to its substrate *in vitro* and to telomeric chromatin *in vivo*." Please note that the entire paragraph was restructured and expanded based on the editor's suggestion (see above).

-p6, paragraph 1 - Sentence "Exhibiting similar..." should be re-formulated.

We have changed this sentence to "Exhibiting similar binding behavior as TRF1, HOT1 was not enriched on any of the subtelomeric variant repeats TCAGGG, TGAGGG and TTGGGG nor on the *C.elegans* telomere TTAGGC repeat sequence."

-p6, paragraph 2 - "To further understand..." rather than "To further manifest..."

This has been changed accordingly.

-p7 - residue K335 mutation to Ala seems to switch sequence specificity to non-telomeric DNA binding; is it possible that this residue is essential for the specificity to telomere binding?

Indeed, Lysine 335 appears to be a key residue for specific recognition of telomeric DNA. As illustrated in Fig. 2c, 2d and 2f the ε-amino group of K335 makes a bifurcated hydrogen bond with carbonyl oxygens of two adjacent guanine bases (the second and third guanines in the TTAGGG motif; G8 and G9 in our DNA

construct) in the major groove. Therefore, this residue is strongly contributing to the recognition of adjacent guanines. This central telomeric characteristic is removed in our scrambled control sequence (GTGAGT) and might thus explain the "gain of function" in the K335 mutant.
We have extended the description of the crystal structure and in particular of the K335 mutant in the results section.

-p9 - one possible explanation for the observed lack of HOT1 association with all telomeres is that some HOT1 foci localize to very short telomeres which are below the limit of detection by FISH or TRF1 immunofluorescence. Telomeric DNA may be completely eroded in these telomeres, at the same time these may be the telomeres marked for elongation by telomerase.

We appreciate this comment by the reviewer and indeed this is a possible explanation. However, we clearly detect telomeres by FISH or TRF1 immunofluorescence that lack detectable HOT1 signal. Given that the degree of association strongly varies between different cellular contexts, we think that a reasonable, alternative explanation is that the access of HOT1 to telomeres itself is regulated by e.g. a post-transcriptional modification. We now elaborate in more detail on this possibility in the discussion section.

-Fig. 3b: helix 3 referred to in p8 should be labelled as in Fig. 2c

Fig. 3b has been labeled accordingly.

-Discussion should mention that HOT1 was previously identified (Dejardin and Kingston, 2008) and how the current study furthers the understanding of its function at telomere.

This has been included both in the introduction and in the results section.

Referee #3 (Remarks to the Author):

The authors have determined that the previously identified HOT1 protein is a double stranded telomere binding protein that is involved in telomere length regulation via regulating access of telomerase to the telomeres during telomere elongation. The data presented in this manuscript provides novel insights into the mechanism of telomere length regulation by telomerase; it raises important questions regarding regulation of telomerase access to the telomeres and enriches the telomere field significantly. There are, however, several issues listed below that need to be addressed before accepting the manuscript for publication in EMBO.

MAJOR ISSUES
Title: Change to "HOT1, a novel vertebrate telomere binding protein recruits telomerase to telomeres"

We have changed the title to "HOT1 is a mammalian direct telomere repeat binding protein contributing to telomerase recruitment" (please also see the comments from the editor and our response).

CST complex dependent telomere maintenance in vertebrates needs to be discussed in the manuscript by the authors. They refer to the shelterin complex as if this is the only complex involved in telomere maintenance

We have extended our introduction and included information on the CST complex in telomere maintenance and, most relevant to this study, its role in termination of telomerase-based telomere extension. The CST complex is now mentioned along with the shelterin complex in the abstract.

Page 6, first paragraph: "HOT1 contains a homeobox domain (Chen et al, 2006), suggesting that it may bind DNA directly." This protein was first identified by Chen et al and the authors showed that HMBOX1 is a transcription repressor. The authors need to modify their abstract and/or Introduction sections of the manuscript accordingly

We have included previous data on HOT1 as a putative transcriptional repressor in our introduction.

Tables: Table of x-ray crystal data collection and refinements statistics is missing

We now provide crystallographic statistics in Table 1 and the structure is deposited under the PDB code 4J19.

Discussion section, page 14, pargarph 2: "The putative mechanism may involve recruiting telomerase-containing Cajal bodies to telomeres, and thus promoting telomerase association with telomeres, perhaps through additional interactions involving TPP1".
This is an important aspect of this work as it has been well established that TPP1 of the shelterin complex recruits telomerase to the telomeres. The research findings presented here raise several important questions: A) Does HOT1 act alone in recruiting telomerase to the telomeres and if so, is this cell cycle dependent? B) Is it possible that HOT1 and TPP1 together coordinate telomerase recruitment to the telomeres and why other screens haven't picked up this interaction previously? C) Is HOT1 an integral component of the Shelterin complex? D) Is there a direct contact between TPP1 and HOT1?

Although both HOT1 and TPP1 seem to contribute to telomerase recruitment, our current data rather suggests that there is no interaction between these two proteins (or between HOT1 and the shelterin complex in general). We had already performed immunoprecipiations with both a mouse and a rabbit anti-HOT1 antibody with nuclear extracts from HeLa cells. While we retrieved our bait HOT1 with SILAC ratios indicating specific enrichment along with the reported interaction partners (Fig. 5, Suppementary Fig. S6, Supplementary Tables 3-6) we have never detected any of the shelterin components. As an additional validation of these results we have now performed immunoprecipitations with SILAC labeled nuclear extracts from mouse embryonic stem cells (in which HOT1 localizes more frequently to telomeres; Fig. 4b). Again, HOT1 was enriched along with dyskerin complex members, Ku70/Ku80 and Cajal body components, but we did not detect any shelterin member (new Supplementary Fig. S6b and Supplementary

Table 7). In a complimentary approach we performed immunoprecipitations with a stable HeLa POT1-LAP cell line (Poser et al., Nat Methods, 2008; LAP: localization and affinity purification tag; includes EGFP), expressing POT1 at endogenous levels. As expected, using SILAC labeled nuclear protein extracts, all members of the shelterin complex were enriched. In agreement with the various HOT1 immunoprecipitations, HOT1 was not detected in the POT1-IP (new Supplementary Fig. S6c and Supplementary Table 8). Similar immunoprecipitation experiments with LAP cell lines for other shelterin members were performed as part of a global proteomic study including hundreds of target proteins and again HOT1 was never identified as an interaction partner for any shelterin member (unpublished data).

These results already provide an explanation for why previous screens have failed to identify a putative interaction between HOT1 and TPP1 or HOT1 as a telomeric protein altogether. Most studies used shelterin components as a starting point, either by immunoprecipitations (Nittis et al., Mol Cell Proteomics, 2010; Giannone et al., PLoS ONE, 2010) or by bimolecular fluorescence complementation (Lee et al., Mol Cell Proteomics, 2011). None of these studies has identified HOT1. While absence of interaction cannot ultimately be proven, this wealth of data suggests that HOT1 is neither an integral component of the shelterin complex nor does HOT1 directly interact with TPP1.
It is noteworthy that from the various global approaches HOT1 was only identified as a telomere-associated factor with the PICh technology (Dejardin and Kingston. Cell, 2009) - like in our study based on DNA and not on shelterin components as bait.

How can both proteins contribute to telomerase recruitment without interacting with each other? While we do not know at present whether HOT1 acts together with additional factors and whether this is cell cycle regulated, it is likely that HOT1 acts at a different step in telomerase recruitment than TPP1. Previous reports have shown that POT1-TPP1 act as a processivity factor for telomerase (e.g. Wang et al., Nature, 2007; Latrick and Cech, EMBO J, 2010; Zaug et al., Genes Dev, 2010). Enhanced *in vivo* processivity would also result in an increased residence time at telomeres, thus explaining the reduction of telomerase-telomere association in TPP1 knock-down/knock-out conditions. In addition, HOT1 might act upstream of TPP1. Given that HOT1 preferentially localizes to telomeres in settings of high *in situ* telomerase activity, one possibility is that HOT1 contributes to an 'open' telomere state that allows/promotes telomere elongation. In such a view, HOT1 might contribute to rendering telomeres accessible and/or contributes to the delivery of telomerase to such telomeres. In return, TPP1 might contribute more prominently to maintaining telomerase at telomeres and thus processivity stimulation. Undoubtedly, the mechanistic contribution of both factors and potential genetic interactions between HOT1 and TPP1 are exciting avenues for future research.
A discussion of the points raised above have been included into the discussion section of the revised manuscript.

MINOR ISSUES
Abstract first sentence: "Telomeres are repetitive DNA structures that together with the shelterin complex protect the ends of chromosomes." There is now evidence, which supports telomere protection by the shelterin and CST complex in vertebrates

We have included this point into the abstract (please also see above).

Title and Abstract: Authors need to specify which organism or species are they referring too early on. Reason: Cdc13 recruits telomerase to the telomeres in yeast and TPP1 in vertebrates

We have changed the title to "HOT1 is a mammalian direct telomere repeat binding protein contributing to telomerase recruitment" (please see above).

End of Introduction section: Re-write sentence: "In addition, we show that HOT1 associates with the active telomerase complex and characterize HOT1 to be localized to telomeres in settings of active processing."

We have re-formulated this content, also incorporating our new data on TERT chromatin binding as follows: "*In vivo*, HOT1 localizes to a subset of telomeres with a higher degree of HOT1-telomere association in cellular contexts of elevated telomere processing. In addition, we show that HOT1 associates

with the active telomerase complex and that HOT1 is required for telomerase chromatin binding."

Results section, page 6, first paragraph: "To determine whether HOT1 was detected in our assay because of a strong association with the shelterin complex or direct binding to TTAGGG repeats, we performed DNA binding assays with HOT1 in vitro."
The authors do not discuss shelterin association of HOT1 in this section of the results so it needs to either be taken out or the appropriate data shown and discussed.

The sentence was shortened accordingly to "To determine whether HOT1 was detected in our assay due to direct binding to TTAGGG repeats, we performed DNA binding assays with HOT1 *in vitro*."

Results section, page 6, last paragraph: "The three longer constructs Q144-A345, L156-A345 and G233-A345 all bound to immobilized telomeric dsDNA baits .... "
What is the binding affinity of HOT1 for double stranded telomeric DNA?

We determined the binding affinity of the HOT1 homeodomain to double stranded telomeric DNA as 800nM, which is in the range of the TRF1 and TRF2 homeodomains, which have published Kd values of 200nM and 750nM, respectively (Hanoaka et al., Prot Sci, 2005).

Figure 2a. What concentration of DNA probes and protein were used?

The biotinylated DNA oligonucleotides were coupled to the paramagnetic streptavidin beads prior to incubation with the extract. The binding capacity for Dynabeads C1 is described with around 20 mg dsDNA per ml. As we added the biotinylated DNA in excess around 1.5 mg of double-stranded telomeric probe (maximum binding capacity) was used for each single pull-down. This corresponds to the 750 µg of beads described in our methods section. For the experiment in Figure 2a, we used 20 µl supernatant of crude *E. coli* lysates (3 mg/ml) containing the overexpressed protein of interest. We have edited the method section accordingly.

Figure 2C: Authors should label the protein and nucleic acid residues in Figure 2C

Fig. 2c has been modified accordingly.

Acceptance letter 15 April 2013

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

Thank you again for this nice contribution to The EMBO Journal, including your additional thorough efforts in the revisions, and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Referee #1

(Remarks to the Author)
The data on Hot1-deleted MEFs clearly illustrate that HOT1 is required for telomerase recruitment. This, together with the result that the homeodomain mediates HOT1 in vivo telomere binding significantly strengthen the paper.
I am happy with the revised version and certainly recommend it for publication.

Referee #3

(Remarks to the Author)

The authors have addressee my questions satisfactorily including the structural analyses of the protein-nucleic acid complex so I am in support of publication of the manuscript.

