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TRF2/RAP1 and DNA-PK mediate a double protection against joining at telomeric ends

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1st Editorial Decision 29 October 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

As you will see from the comments enclosed below, all expert referees appreciate the additional insight provided by your study proposing the involvement of alternative NHEJ at telomeres. However, these scientists also request necessary improvements related to data quality and quantification (ref#2) and the use of additional substrates to unambiguously discriminate between aNHEJ or inefficient cNHEJ to account for the reported effects (ref#1). Further, ref#3 recommends to significantly develop or discard the currently rather preliminary results on PARP-1/Mre11. Conditioned on these clearly essential improvements we would be delighted to assess a revised version of your paper in the near future. I also have to remind you that it is EMBO J policy to allow a single round of major revisions only, and that the final decision on acceptance or rejection entirely depends on the content within the final version of your manuscript!

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. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Bombarde et al. utilizes an in vitro plasmid based assay similar to the one originally published by the Baumann lab to analyze the contributions of telomeric and NHEJ-dependent proteins in telomere end protection. They find that the TRF2/RAP1 complex inhibits loading of DNA-PK at telomere ends to inhibit NHEJ-mediated fusions. Interestingly, they uncover a role for DNA-PK at telomeres by inhibiting the alternative NHEJ (aNHEJ) pathway, a Lig4 independent repair pathway. They postulate a "double lock" mechanism of regulation at telomeres. These results are of interest and are the first to point to aNHEJ as a possible repair pathway at telomeres. However, there are several issues that must be resolved.

Comparing the data from the Baumann paper (Mol Cell 2007) reveals several discrepancies. In the Baumann paper, depletion of KU yielded no ligated products (no dimer or higher multimer formation), consistent with the requirement for NHEJ in mediating DNA end joining. However, in the current manuscript, a distinct dimer is still observed in extracts depleted of XRCC4 (Fig. S1). My question is: why is the dimer observed when NHEJ factors are depleted? Is this due to poor XRCC4 depletion? This dimer is also observed in pT3' plasmids with telomeric sequence near the end, contrary to data in the Baumann paper in which terminal telomeric sequences protects against NHEJ-mediated end joining. These data are troublesome, since the authors use this dimer formation assay in KU/LIG4 depleted extracts to claim that telomere ligation occurs independent of NHEJ (and hence must take place via aNHEJ). An explanation of these diseperate results is required.

If dimer formation in KU/Lig4/PK depleted extract is really due to aNHEJ, then this activity is either very inefficient or does not efficiently process blunt DNA ends used in this study (aNHEJ has been known to require short single stranded homology regions). The authors need to generate plasmids containing 3' single stranded overhangs to further confirm whether ligation is due to aNHEJ (as they claim) or inefficient cNHEJ (due to inefficient depletion of NHEJ proteins in their extracts).

Finally, the English usage is often very confusing (for example, what are "clue genes"?)-this paper could use some editing by a native English speaker. The "double lock" used in the title should also be modified.

Referee #2 (Remarks to the Author):

In the present paper the authors address the long standing question of the mechanism that prevents end joining between telomeres, as well as the role of NHEJ components found in the telomeric regions. Evidence is presented that TRF2/RAP1 and DNA-PK mediate a double lock protection against end joining at this site of the chromosome.
A long standing conundrum in the field of telomere research is how to reconcile the presence at these structures of DNA double strand break repair proteins such and Ku and DNA-PKcs and to explain the known protective role they exert in the maintenance of telomeres, when they can actually catalyze their destruction though end joining. While a significant amount of work has been carried out in the field and a bulk of important data has become available, available data are partly contradictory and cannot be organized in a comprehensive model.
Here the authors present a biochemical study designed on the basis of recent developments regarding DSB repair pathways that allows them to generate a compelling model of the protection mechanisms operating at the telomeres. Using an in vitro assay and a relevant DNA plasmid substrate the authors provide evidence for functional inhibition of DNA-PK by the TRF2/RAP1 complex that prevents the classical DNA-PK dependent pathway of NHEJ (which the authors term C-NHEJ) to operate at the telomeres. In addition, using recent evidence for alternative or backup

end joining (which the authors term B-NHEJ) the authors provide evidence that inhibition of this mode of end joining is afforded by Ku and is potentiated by DNA-PKcs. These discoveries allow the development of a nice model for telomere protection based on a hierarchy starting with TRF2/RAP1 and continuing with DNA-PK. It allows putting the majority of available data in this topic into perspective and offers an elegant way for overcoming apparent contradictions.
The results presented are novel, significant for the field and merit publication in the Journal pending revision as suggested below. The paper is concise and well written and the experiments presented appear to have been conducted with care and attention to the detail.

Specific Comments

1. Telomeres normally have tails of single stranded DNA participating in the T-loop formation. The substrate used by the authors lacks this structure for obvious reasons. Although blunt telomeres can be generated at times in the cellular environment, it is likely that the single stranded structure will remain prominent most of the time. Have the authors considered examining the function of their assay using substrates with slightly longer overhangs to study their effect of C-NHEJ and B-NHEJ, as well as on the effect of TRF2/RAP1 in suppressing C-NHEJ? A short discussion on this subject, as well as possibly available experimental evidence will be useful to the interested reader.
2. The reaction conditions employed in the in vitro plasmid assay used are not physiological and have been developed with the purpose of developing an assay reflecting DNA Ligase IV function. This is actually done by artificially suppressing the function of other DNA ligases. While this is fine when staying in the framework of C-NHEJ it becomes problematic when testing interactions between C-NHEJ and B-NHEJ, as the latter will function much more efficiently under more physiological conditions of Mg2+ etc. Have the authors tested their conclusions at other reaction conditions? Discussion of the subject and, if available, presentation of such results will strengthen the manuscript.
3. Some of the results shown in Figure 2B, but also in several other Figures throughout the manuscript, only show very faint signals for the end joining products, barely visible in the print out. It will help the reader if the authors could improve the quality of the photographs to clearly show the detected products. Also quantification of the results presented similar to that shown in Figure 1D will enhance the power of the conclusions drawn and will help the reader to rationalize the interpretation given.
4. The results presented in Figure 2 after IP of TRF2/RAP1 suggest a significant reduction in the overall end joining activity of the extract, possibly a consequence of the associated manipulations. This aspect will need to be discussed, some explanation will need to be provided and quantification should be added to offer a quantitative evaluation of the effect. Also the quality of the included picture will need to be improved in order better show the products obtained and their variation between the different reactions assembled. Furthermore, the DNA-PKcs band in Figure 2D shows two bands, an effect not so prominent in other experiments. Have the authors an explanation this?
5. Several of the product signals in Figure 3 are barely visible. The authors should include improved images with the paper.
6. Together with the added quantification suggested above, the authors should also provide information on the reproducibility of the results obtained and when relevant estimates for the errors associated with the measurements shown. This is already done for certain measurements but should be extended to the majority of the results presented.
7. Page 6, last sentence of third paragraph: The conclusion drawn here is correct only in the context of the reaction conditions used, which suppress the function of ligases other than DNA Ligase IV. Have the authors tested how this reaction will fare at higher concentration of Mg2+?
8. Page 7, para 4. Here again similar arguments can be formulated. While Ku appears to prevent DNA Ligase III from operating under these reaction conditions, published biochemical data also suggest that Ku assist the functions of DNA Ligase III. A convincing argument in favor of the interpretation given by the authors is the comparison of the in vivo effects of Ku and LIG4 deficiency. However, some discussion of the point and a couple of words of caution should be included in this place.
9. The paper will benefit from some editing.

Referee #3 (Remarks to the Author):

This manuscript describes an in vitro approach to understand how C-NHEJ vs A-NHEJ is being regulated at the telomere end to prevent end fusion. This is an important question which hasn't been clearly understood at this moment in mammalian cells. In this manuscript, this group has adapted a novel telomere substrate to mimic telomere ends in vivo and used human cell lysate in combination with immunodepletion to verify factors that would modulate C-NHEJ and concluded that TRF2/RAP1 prevent C-NHEJ mediated end-fusion in the presence of DNA-PK complex. The results are quite convincing and provide important information for a potential mechanism in preventing DNA end fusion at telomere. However, the suggestion of the involvement of PARP-1 and Mre11 based on the pull down experiment is premature. It may require alternative approach to further verify the observation.

Specific comments:

1. As a control, I would suggest to knockdown TRF1, TRF2 and RAP1 individually. Since knock down any of the telomere binding proteins would affect the complex, it would be important to show that there is no effect by TRF1.
2. It is not clear why 75 vs 150 mM salt concentrations were used in the assay. Recent publication verifies that DNA-PKcs is recruited to DSBs by Ku in vivo. Therefore, end join assay involved in Ku/DNA-PKcs should be done in a salt concentration mimic reflect physiological condition.
3. As discussed earlier that pull down of Mre11 and PARP is interesting but requires direct evidence to show their involvement and function at telomere.

1st Revision - authors' response 10 February 2010

Reply to Referee 1

Comparing the data from the Baumann paper (Mol Cell 2007) reveals several discrepancies. In the Baumann paper, depletion of KU yielded no ligated products (no dimer or higher multimer formation), consistent with the requirement for NHEJ in mediating DNA end joining. However, in the current manuscript, a distinct dimer is still observed in extracts depleted of XRCC4 (Fig. S1). My question is: why is the dimer observed when NHEJ factors are depleted? Is this due to poor XRCC4 depletion?

Actually, the experiment referred to by the reviewer (Fig S1; see also Fig S3D) concerns inhibition of C-NHEJ on a control non-telomeric substrate by addition of blocking polyclonal antibodies raised against XRCC4 (and not depletion of XRCC4) which strongly inhibited the reaction although a faint ligated product was still detected. This result is very reminiscent of the one obtained in Baumannís paper in which the addition of blocking antibodies against KU strongly inhibited the ligation with the scrambled plasmid but still allowed a faint amount of dimer to be produced (Bae et al, Fig 1D). Similarly, in Bae et al, a distinct dimer is still produced with the scrambled plasmid even after KU depletion and despite a degradation of the substrate (Bae et al, Fig3B lane 7).

To more confidently attribute the reaction to C-NHEJ, we additionally inhibited the reaction with the specific inhibitor of DNA-PKcs, NU7026 (Figure 1B, lane 9; Figure S1, lane 2; Figure S2, lanes 4-9) (evidence more direct than in Bae et al in which only the non-specific inhibitor wortmannin was used - therein, Fig 1C). Moreover, we showed that extracts completely devoid of DNA ligase IV (not by depletion but due to targeted disruption in both ligase IV alleles in cells) exhibited nearly complete disparition of the ligated product of the control plasmid (Figure 3B, lane 1; Fig S3B, lane 2).

We agree that under all these various C-NHEJ inhibiting conditions, a distinct dimer was still observed (about 2% ligation activity as quantified in Fig 1B). A possibility is that it may be due to non C-NHEJ activity (hereafter named B-NHEJ) marginally operating on a minor fraction of the plasmid, even in the presence of KU. This interpretation is supported by the slight stimulating effet of beta-NAD observed on the faint residual ligation activity in KU+/LIG4- extracts (quantified in Fig 3C, IgG only-depleted extracts). Another argument supporting this interpretation is developped in the response to the following statement of the reviewer.

This dimer is also observed in pT3' plasmids with telomeric sequence near the end, contrary to data in the Baumann paper in which terminal telomeric sequences protects against NHEJ-mediated end

joining.

In Bae et al, the plasmid used could be ligated by both head or tail ends so that no condition in their paper led to complete inhibition of dimer formation (see Fig 2 therein; also observed in our paper with similar plasmids in Fig 1B, lanes 1-2, 5-6 and in Fig S1B), impairing a precise estimation of a possible remaining activity on the telomeric end. To avoid multimeric ligation in addition to the dimeric form, we have blocked the end opposite the telomeric sequence by a biotin residue (Fig 1B), so that inhibition of the ligation efficiency could be easily quantified by the yield of dimer production (80% inhibition on the telomeric sequence, as quantified in Fig. 1B). Since we showed later that B-NHEJ was insensitive to TRF2/RAP1 inhibition (Fig 4B), a reasonable interpretation of the marginal ligation activity observed on telomeric ended plasmids is B-NHEJ operating a *minima* and despite the presence of KU. This minimal B-NHEJ activity could also account for the slight residual ligation activity observed under conditions of C-NHEJ inhibition (XRCC4 antibodies, DNA-PK inhibitor or Lig4- extracts) and despite the presence of KU (see previous paragraph).

These data are troublesome, since the authors use this dimer formation assay in KU/LIG4 depleted extracts to claim that telomere ligation occurs independent of NHEJ (and hence must take place via aNHEJ). An explanation of these diseperate results is required.

We attributed to B-NHEJ the ligation activity observed at telomeric ends after depletion of KU or KU/DNA-PKcs from Lig4-defective extracts on the basis of a convergent set of evidence that clearly distinguished this activity from C-NHEJ :

- strongly enhanced after removing KU (the keystone protein of the C-NHEJ reaction) in Lig4-null extracts (de facto C-NHEJ defective) (Fig 3B and 3C)
- insensitive to addition of a DNA-PKcs inhibitor (Fig S3C)
- insensitive to addition of blocking antibodies against XRCC4 (Fig S3D)
- inhibited upon KU addition (Fig3 D and E),

all these characteristics being exactly opposite to what would be expected from a C-NHEJ KU-dependent ligation.

In Bae et al, the authors also depleted the extracts from KU but observed a degradation of the substrate rather that ligation (Bae et al, Fig 3B), whereas in our case we revealed a new ligation actvity with no degradation of the substrate (Fig 3B). This may be due to differences in preparation of the extracts.

aNHEJ has been known to require short single stranded homology regions. The authors need to generate plasmids containing 3′ single stranded overhangs to further confirm whether ligation is due to aNHEJ (as they claim) or inefficient cNHEJ (due to inefficient depletion of NHEJ proteins in their extracts).

From the set of evidence listed above, we confidently believe that the activity observed after KU depletion was not due to inefficient C-NHEJ. However, to definitively differentiate C-NHEJ and the ligation observed upon KU-depletion in Lig4-nul extracts, we have performed the experiment required by the referee.

As stated by the referee, we reasonned that C-NHEJ would ligate incompatible ends whereas B-NHEJ would much more rely on complementary ends for ligation. Thus we compared the ligation products obtained with a pBS substrate bearing non compatible ends (HindIII/ BamHI) or compatible ends (HindIII). It has been already shown that decreasing the plasmid concentration favors intramolecular ligation (e.g. (Teraoka & Tsukada, 1987)). So we lowered plasmid concentration and performed ligation under otherwise identical conditions as described under the material and methods section for either C-NHEJ (Lig+ IgG-depleted extracts) or B-NHEJ (Lig4-KU-depleted extracts) (additional Figure 1). After migration without intercalating dye and then staining with SYBR-Green (add. Figure 1, left gel, lane 1), novel species were visualized in addition to dimer (D) and multimers (M) that corresponded to relaxed circle (RC) migrating slower than the linear monomer (LM) and supercoiled circles (SC) with various topoisomers migrating faster than the linear monomer and most likely due to topoiomerase activity in the extracts. When the same ligation products were run in the presence of BET (add. Figure 1, right gel, lane 1), topoisomers were resolved as a single SC band as expected due to the dye intercalation.

SC and RC forms came from intramolecular ligation and thus could be used to assess the capability of the extracts to ligate non compatible (nonC) or compatible (C) ends depending on the plasmid substrate used. Under C-NHEJ conditions, non compatible and compatible ends yielded the same amount of intramolecular ligation products (add. Figure 1, lanes 1-2 and 5-6). Under B-NHEJ conditions and in striking contrast, SC and RC intramolecular ligation products were absent with non-compatible ends, although dimers were still produced as expected (add. Figure 1, lanes 3 and 7),

whereas intramolecular reaction occurred normally with compatible ends (add. Figure 1, lanes 4 and 8).

These data clearly confirmed that B-NHEJ mostly rely on microhomology. In addition to the other characteristics detailed above, we consider that these results unambiguously differentiate from C-NHEJ the ligation activity exhibited under KU depletion in Lig4- extracts. Since we plan to develop this approach with compatible or incompatible ends to further characterize B-NHEJ, we have mentionned briefly these results as data not shown in the manuscript (page 8, end of 2nd paragraph). However we could add them as supplementary figure if requested by the referee.

Finally, the English usage is often very confusing (for example, what are "clue genes"?)-this paper could use some editing by a native English speaker. The "double lock" used in the title should also be modified.
Editing of the ms by an american scientist, Dr N Johnson, PhD, has been conducted. The double lock was removed from the title.

References:

Teraoka H, Tsukada K (1987) Influence of polyethylene glycol on the ligation reaction with calf thymus DNA ligases I and II. J Biochem 101(1): 225-231

Tsai YC, Qi H, Liu LF (2007) Protection of DNA Ends by telomeric 3' G-Tail sequences. J Biol Chem 282: 18786-18792

Reply to Referee 2

1- Telomeres normally have tails of single stranded DNA participating in the T-loop formation. The substrate used by the authors lacks this structure for obvious reasons. Although blunt telomeres can be generated at times in the cellular environment, it is likely that the single stranded structure will remain prominent most of the time. Have the authors considered examining the function of their assay using substrates with slightly longer overhangs to study their effect of C-NHEJ and B-NHEJ, as well as on the effect of TRF2/RAP1 in suppressing C-NHEJ? A short discussion on this subject, as well as possibly available experimental evidence will be useful to the interested reader.

We agree with the referee that our substrate does not match perfectly the physiological structure of telomeres, that is a 3í telomeric tail which could aditionally adopt a G-quadruplex conformation. We intented first to check the balance between C- and B-NHEJ on a simpler model. Such a substrate has not been much used in the literature due to obvious technical reasons and we have no currently available data on end-joining with a more sophisticated 3í protruding substrate. However, to follow the referee's suggestion, we have added a short discussion regarding this point in the Discussion section (page 11, beginning of the last paragraph) and added a reference which directly adressed the effect of G-quadruplex on DNA-PK activation (Tsai et al, 2007).

2- The reaction conditions employed in the in vitro plasmid assay used are not physiological and have been developed with the purpose of developing an assay reflecting DNA Ligase IV function. This is actually done by artificially suppressing the function of other DNA ligases. While this is fine when staying in the framework of C-NHEJ it becomes problematic when testing interactions between C-NHEJ and B-NHEJ, as the latter will function much more efficiently under more physiological conditions of Mg2+ etc. Have the authors tested their conclusions at other reaction conditions? Discussion of the subject and, if available, presentation of such results will strengthen the manuscript

We acknowledge the referee to point out this important issue since it had inadvertently disappeared from the first version of the manuscript. Actually, we were aware of the different Mg2+ concentration requirement of C- and B-NHEJ thanks to the work of Iliakisís group who carefully examined the biochemical requirements of both activities *in vitro* (Wang et al, 2001). So we performed a ligation reaction with the Lig4-/Ku-depleted extracts, conditions corresponding to B-NHEJ and we varied the Mg concentration from 0.5 to 5 mM, in the presence or not of 2 mM NAD. As shown in additional Figure 2, the yield of dimer production increased 2 fold and remained stimulated by NAD up to 3 mM Mg. Therefore, we standardized the Mg concentration to 3 mM in the presence of 2 mM NAD for B-NHEJ conditions. This point is now integrated in the material methods (end-joining assay) and the results section (page 8, second paragraph), the results in

additional Figure 2 are mentionned as data not shown and the Iliakisís paper is added to the references.

3-1/ Some of the results shown in Figure 2B, but also in several other Figures throughout the manuscript, only show very faint signals for the end joining products, barely visible in the print out. It will help the reader if the authors could improve the quality of the photographs to clearly show the detected products.

Corrections have been made to improve the visibility of the data.

3-2/ Also quantification of the results presented similar to that shown in Figure 1D will enhance the power of the conclusions drawn and will help the reader to rationalize the interpretation given. and 6- Together with the added quantification suggested above, the authors should also provide information on the reproducibility of the results obtained and when relevant estimates for the errors associated with the measurements shown. This is already done for certain measurements but should be extended to the majority of the results presented.

For all the ligation or DNA-PK activity experiments quantified (Fig 1C, 2B, 3B, 3D), the experiments were performed at least three times and the quantifications are presented (Fig 1D, 2C, 3C, 3E) with error bars as SEM. For two end-joining reactions in the paper (Figure 1B and Figure 4B), the experiments were done twice with very similar results. The second experiment for each Figure 1B and Figure 4B are presented as Additional Figures 3 and 4, respectively. We have added quantification of the representative figures in their respective legends (Fig 1B and Fig 4B).

4- 1/ The results presented in Figure 2 after IP of TRF2/RAP1 suggest a significant reduction in the overall end joining activity of the extract, possibly a consequence of the associated manipulations. This aspect will need to be discussed, some explanation will need to be provided and quantification should be added to offer a quantitative evaluation of the effect.

The immunodepletion procedure was optimized as follows briefly : overnight incubation of the mixture (extracts+ agarose beads+antibodies) in the minimal volume at 4^{o}C under constant rotation, spinning, mixing again with clean beads, incubation for 1 hr at 4^{o}C and spinning again to remove the beads All these manipulations obviously decrease the overall efficiency of the ligation reaction, possibly relying on partial protein inactivation, non -specific loss on the agarose beads and slightly dilution of the extracts during the IP-procedure. The absolute ligation efficiency after control immunodepletion was 4.6 ± 0.8% SEM (n=3) on the control plasmid. However, as quantified in Figure 2C, the end-joining activity of TRF2/RAP1-depleted extracts was conserved, as compared to control IgG-depleted extracts on the control biopC plasmid (92.5 % ± 4% SEM, n=3) whereas the ligation efficiency actually increased 9-fold on the telomeric substrate with these extracts, indicative of a very specific effect of TRF2/RAP1 depletion on the telomeric substrate. A sentence has been added in the manuscript to point out the decrease in the overall end-joining activity after immunodepletion (page 6, last paragraph) and the ligation efficiency is given in the legend of Figure 2C.

4-2/ Also the quality of the included picture will need to be improved in order better show the products obtained and their variation between the different reactions assembled.
Please see above point 3-1 the response to a similar statement

4-3/ Furthermore, the DNA-PKcs band in Figure 2D shows two bands, an effect not so prominent in other experiments. Have the authors an explanation this?

HeLa cells extracts used in Fig. 2D and 2E were not from the same batch and we irregularly observed in some batches on low % agarose gels an extra band migrating lower than full length DNA-PKcs and which could originate from limited proteolytic digestion, although without loss of activity.

5- Several of the product signals in Figure 3 are barely visible. The authors should include improved images with the paper.
Please see above point 3-1 the response to a similar statement

7- Page 6, last sentence of third paragraph: The conclusion drawn here is correct only in the context of the reaction conditions used, which suppress the function of ligases other than DNA Ligase IV. Have the authors tested how this reaction will fare at higher concentration of Mg2+? and 8- Page 7, para 4. Here again similar arguments can be formulated. While Ku appears to

prevent DNA Ligase III from operating under these reaction conditions, published biochemical data also suggest that Ku assist the functions of DNA Ligase III. A convincing argument in favor of the interpretation given by the authors is the comparison of the in vivo effects of Ku and LIG4 deficiency. However, some discussion of the point and a couple of words of caution should be included in this place.

Our conclusion that ligation on telomeric DNA in the absence of TRF2/RAP1 relied on C-NHEJ was based on its sensitivity to DNA-PKcs inhibitor but also on the fact that ligation was abolished in extracts from Lig4-null cells (Fig S3B, lane 8). We have not directly tested the effect of increasing Mg2+ concentration in normal extracts on ligation with telomeric DNA but we doubt that it would shift to ligation dependent on another ligase. From the results presented in our paper and reports from the litterature (Guirouilh-Barbat et al, 2007; Schulte-Uentrop et al, 2008; Wang et al, 2006), we rather believe that the main inhibitor of the alternative ligation route is KU. Indeed, when we assessed ligation activity with Lig4-null extracts under high Mg2+ concentration, no ligation was oberved either on control or telomeric DNA unless KU was depleted from the extracts (additional Figure 5). In addition, the ligation activity observed at telomeric ends after depletion of KU or KU/DNA-PKcs from Lig4-defective extracts was actually inhibited upon KU addition (Fig3 D and E).

It has been reported that under ligation conditions with purified mammalian ligases and short duplex oligonucleotides, KU could stimulate any of Ligase I, III or IV depending of the DNA ends, mostly via a end-bridging activity (Ramsden & Gellert, 1998). However, the relevance of this activity in cells has not been demonstrated yet. On the opposite, cellular arguments rather favor a competing role of KU against alternative repair pathway, including B-NHEJ, Single-strand annealing and homologous recombination (Schulte-Uentrop et al, 2008). As stated by the referee, striking evidence in vivo and in cellulo include the rescue of Lig4-null mice lethality by a supplementary knockout of KU (Karanjawala et al, 2002) and also the increase in survival of Lig4-deficient cells to ionizing radiation by a secondary inactivating mutation in KU (Adachi et al, 2001). As suggested by the referee, the in vitro and in vivo references have been added together with a short discussion of the point (page 11, first paragraph).

9. The paper will benefit from some editing.

Editing of the ms by an american scientist, Dr N Johnson, PhD, has been conducted.

References:

Adachi N, Ishino T, Ishii Y, Takeda S, Koyama H (2001) DNA ligase IV-deficient cells are more resistant to ionizing radiation in the absence of Ku70: Implications for DNA double-strand break repair. Proc Natl Acad Sci U S A 98(21): 12109-12113

Guirouilh-Barbat J, Rass E, Plo I, Bertrand P, Lopez BS (2007) Defects in XRCC4 and KU80 differentially affect the joining of distal nonhomologous ends. Proc Natl Acad Sci U S A 104(52): 20902-20907

Karanjawala ZE, Adachi N, Irvine RA, Oh EK, Shibata D, Schwarz K, Hsieh CL, Lieber MR (2002) The embryonic lethality in DNA ligase IV-deficient mice is rescued by deletion of Ku: implications for unifying the heterogeneous phenotypes of NHEJ mutants. DNA Repair (Amst) 1(12): 1017-1026

Ramsden DA, Gellert M (1998) Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks. EMBO J 17(2): 609-614

Schulte-Uentrop L, El-Awady RA, Schliecker L, Willers H, Dahm-Daphi J (2008) Distinct roles of XRCC4 and Ku80 in non-homologous end-joining of endonuclease- and ionizing radiation-induced DNA double-strand breaks. Nucleic Acids Res 36(8): 2561-2569

Tsai YC, Qi H, Liu LF (2007) Protection of DNA Ends by telomeric 3' G-Tail sequences. J Biol Chem 282: 18786-18792

Wang H, Zeng ZC, Perrault AR, Cheng X, Qin W, Iliakis G (2001) Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells. Nucleic Acids Res 29(8): 1653-1660

Wang M, Wu W, Wu W, Rosidi B, Zhang L, Wang H, Iliakis G (2006) PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res 34(21): 6170-6182

Reply to Referee 3

As a control, I would suggest to knockdown TRF1, TRF2 and RAP1 individually. Since knock down any of the telomere binding proteins would affect the complex, it would be important to show that there is no effect by TRF1.

To answer the referee's concern, we have tried to separately deplete TRF1 from the extracts to check for an effect on C-NHEJ inhibition with telomeric plasmid. We have tested three different antibodies, individually or in combination (Calbiochem = rabbit 581420 ; Novus = mouse NB110-6828155 ; Santa Cruz = mouse sc-5596). However, there was either no significant depletion or under conditions of significant TRF1 depletion, a codepletion of TRF2 and RAP1 from the extracts precluded any evaluation of the individual effect of TRF1 (additional Figure 6). Such cross-reaction between antibodies have already been observed and reported in other papers, like Bae et al. who found that TRF1 antibodies codepleted TRF2 (Bae & Baumann, 2007), which is not surprising given the sequence and structural proximity of some of their domains (Chen et al, 2008; Hanaoka et al, 2005).

Nevertheless, we confidently believe that TRF1 does not play a major role in C-NHEJ avoidance at telomeres for the following reasons drawn from our data and reports in the litterature :

- TRF1 was equally pulled-down on the telomeric probes, whether TRF2/RAP1 was present or not (Fig 2E, lanes 4 and 6) ; however, KU end-binding and release of C-NHEJ inhibition on telomeric probe was only obtained after TRF2/RAP1 depletion, namely under conditions where TRF1 still bound to the telomeric probe. We concluded that at ds telomeric ends, TRF2/RAP1 complex but not TRF1 alone is likely responsible for an hindrance of the DNA-PK loading and in turn impairement of LIG4-dependent end-joining at these ends.
- Baumann's group has already demonstrated TRF2/RAP1-mediated end-joining inhibition on telomeric DNA and has shown that addition of excess amount of TRF1 in the absence of TRF2 did not reconstitute this inhibition (Bae & Baumann, 2007); accordingly, the same group has confirmed recently that the major inhibiting factor of NEHJ at telomeres is RAP1 (Sarthy et al, 2009).

It is not clear why 75 vs 150 mM salt concentrations were used in the assay. Recent publication verifies that DNA-PKcs is recruited to DSBs by Ku in vivo. Therefore, end join assay involved in Ku/DNA-PKcs should be done in a salt concentration mimic reflect physiological condition.

As stated by the referee, DNA-PKcs has been demonstrated to be recruited on DNA via KU in cells (Uematsu et al, 2007). In vitro, it has also been shown that most of DNA-PKcs loading to DNA ends requires KU at salt concentration higher than 50 mM (Hammarsten & Chu, 1998). The composition of the ligation buffer that we used is the one commonly employed by most of the groups dealing with end-joining in vitro and originates from a pioneering publication in the field (Baumann & West, 1998) which already employed 60 mM KOAc. Considering the volume of extracts added, the final KOAc concentration in the reaction raises 80 mM which is exactly in the concentration range required to preserve the KU-dependence of DNA-PKcs loading and activation on DNA ends (Hammarsten & Chu, 1998)- that is indeed physiologically relevant.

The reason why we used higher salt concentration for the pull-down experiment (Fig 2D) was to disrupt the KU-TRF1 or TRF2 interactions (Hsu et al, 2000; Song et al, 2000) which would otherwise preclude evaluation of direct KU binding to the probe ends. In addition, such 150 mM salt concentration has been shown to preserve KU-DNA-PKcs interaction (DeFazio et al, 2002).

As discussed earlier that pull down of Mre11 and PARP is interesting but requires direct evidence to show their involvement and function at telomere.

We agree with the referee that the results of the pull-down experiments is far from a direct evidence of MRE11 and PARP1 function at telomeres. However, various approaches have allowed to implicate these proteins in alternative end-joining mechanisms (Audebert et al, 2004; Deriano et al, 2009; Rass et al, 2009; Wang et al, 2006; Xie et al, 2009; Zhuang et al, 2009). In addition, the stimulating effect of beta-NAD on B-NHEJ on telomeric substrate that we observed here suggested that at least PARP1 may be involved (Audebert et al, 2004). Our aim was just to use these two

protein as admitted candidate for alternative end-joining and to check for a competition between KU and their loading at DNA ends, according to models in the litterature (Wang et al, 2006). Since we agree that these results are preliminary, we propose to keep them in the main text body of the manuscript but to delete the corresponding sentence in the abstract. In addition, we have removed PARP1 and MRE11 from the model depicted in Figure 5.

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2nd Editorial Decision 26 February 2010

I just received very positive feedback from one of the original referees on your revised paper. The only request was to indeed incorporate fig.1 that was provided to the referee's only as supplementary information. From an editorial perspective I would kindly ask you to proceed similarly with the referee figures 5 and 6, as these contain relevant information.

As indicated in the initial decision, our transparent review process would usually demand availability of ALL information that contributed to the final decision (i.e. also the figures provided to referees in the responses to their comments). Shifting the above mentioned figures into the papers supplement would satisfy this need, as the residual referee figures (2, 3 and 4) provided in the rebuttal do not add further substantial information.

Please modify the manuscript accordingly and provide as with such a version to your earliest convenience. This will enable efficient official acceptance and timely publication of your study.

Editor
The EMBO Journal

2nd Revision - authors' response 03 March 2010

We have followed all your recommendations and now submit a new version modified as follows : 1/ Additional Figure 1, 5 and 6 initially provided for referees only have been included in the Suppplementary Figures (FigS5, S6 and S3 respectively); 2/ the corresponding figure legends have been included in the Supplementary material; 3/ sentences referring to the corresponding figures have been included in the text body (p6, paragraph 2, refers to Supplemental Fig. S3; p8, first paragraph, refers to Supplemental Fig. S5 instead of data not shown and to Supplemental Fig. S6 in addtion to Fig. 3C).

