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Novel roles for A-type lamins on telomere biology and the DNA damage response pathway

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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 26 March 2009

Thank you for submitting your manuscript to the EMBO Journal. Please find enclosed the comments of two of the three reviewers whom we had asked to review your manuscript for EMBO Journal. We are still waiting for a third report, but given the two present reports I can take a decision now. As you can see below, both referees appreciate the analysis and find the described link between Lamin A function and telomere biology very interesting. While referee #1 has only a minor concern, referee #3 raises a number of different issues that have to be resolved in order for further consideration here. In particular, some of the raised issues concern 53BP1 and some further insight into how loss of LaminA leads to decreased 53BP1 levels and if this is directly linked to the telomere phenotypes observed upon loss of LaminA is needed. Should you be able to address the criticisms of the reviewers in full, we would consider a revised manuscript. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should also add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to address the points raised at this stage. I will forward you the comments of the third referee as soon as we receive them.

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):

This is well-written manuscript that makes an important connection in the study of nuclear organization. The authors report connections between A-type lamin function and telomere biology. They find that cells lacking A-type lamins have altered distribution of mouse telomeres and altered heterochromatic marks at telomeres. Some of these findings can be linked to reduced levels of retinoblastoma protein family members, a known consequence of loss of A-type lamins. Others are independent of Rb family members. These findings are important since mutations in A-type lamins are linked to Hutchinson-Gilford progeria and altered telomere function is a possible link to disease mechanism. Also, loss of A-type lamin expression is linked to tumor progression and altered telomere function may be related to this phenotype as well. Of particular interest is the finding that 53BP1 levels are reduced in cells lacking A-type lamins. This could provide an explanation for altered DNA damage responses associated with laminopathies.
The data presented in this study is generally very sound. These findings will be of considerable interest to a wide readership and will foster further studies. There is one nominal concern (mostly editorial) that, if addressed, would further clarify the manuscript. Differences in the TRF analysis of adult fibroblasts (vs. MEFs) are stated to indicate that telomere defects are exacerbated during aging. This at least in the formal since is an overstatement. The authors should be clear what they mean by the term "aging," since these two populations of fibroblasts may be different for a range of reasons other than normal aging. In addition, telomere length appears to be longer in adult fibroblasts than MEFs, although cross-comparison may be difficult. Is there a reason for this?

Referee #3 (Remarks to the Author):

In this manuscript, the authors investigate the role of A-type lamins on telomere biology and demonstrate that loss of A-type lamins results in altered distribution of telomeres in the nucleus, telomere shortening, reduced tri-methylation of H4K20 at telomeres, and increased genome instability associated with an increase in chromosome number and breaks. Furthermore, uncapping of telomeres in the absence of A-type lamins results in a reduction of telomere fusions (compared to Lmna+/+ cells), which the authors correlate with decreased protein levels of 53BP1. Overall, the manuscript is well written and the work is clearly presented. Furthermore, the data provide evidence for a number of interesting findings linking telomere biology to nuclear organization, and specifically the nuclear lamina. However, the data raise a number of questions and lack several key experiments as outlined below. In light of these issues, I feel that some further work would be needed before I could consider this work suitable for publication in EMBO J.

1) The authors reference several publications demonstrating that cell cycle phase and senescence result in altered telomere positioning within the nucleus. As lamins are known to affect DNA transactions including replication, it is important to show cell cycle profiles (FACS analysis) of Lmna+/+ and Lmna-/- cells to determine whether or not cell cycle differences might contribute to the results presented in Fig 1. Along these same lines, since lamins are important for the stability of Rb, could differences in telomere distribution be related to differences in the percentage of senescent cells between Lmna-/- and Lmna+/+ cells? Experiments examining -gal staining (for example) would address this question.

2) In Fig. 1B and C, the authors find that loss of A-type lamins result in a shift in the localization of telomeres towards the nuclear periphery. In addition, using ChIP experiments they demonstrate that lamins A and C associate with telomeric DNA suggesting that lamins tether telomeres to the nuclear matrix. Wouldn't one expect that if lamins are important for tethering telomeres, that their loss would result in the movement of telomeres to the interior of the nucleus (away from the periphery)?

3) A decrease in tri-methylation of H4K20 is demonstrated in Lmna-/- cells compared to wild-type

(Fig. 3). Is this decrease due to reduced expression of SUV4-20H1 or SUV4-20H2? In addition, cells deficient for SUV4-20H showed reduced levels of H4K20me3 at telomeres, and these changes were accompanied by telomere elongation (Benetti et al., J Cell Biol 178:925-936, 2007). A mechanism (or at least a discussion) needs to be provided as to why reduced H4K20me3 correlates with longer telomeres as described by Benetti et al., and shorter telomeres described in this report.

4) It is not clear why the authors use immunoprecipitation followed by Western blotting to demonstrate loss or reduction of Rb, p130, and p107 in Lmna-/- cells compared to Lmna+/+ cells. Such experiments don't allow for loading controls. It would be better just to prepare lysates (ex. in Laemmli buffer) and then probe for the levels of these proteins by Western blotting. These same lysates could be used to examine levels of SUV4-20H1 or SUV4-20H2, as mentioned above.

5) Increased genome instability is demonstrated for Lmna-/- cells in Fig. 4 and Suppl Fig. 4. Several of these phenotypes, as well as the reduced H4K20me3 levels, are similar to cells lacking Rb family members. It would be interesting to complement and/or overexpress Rb (and/or other family members p107 and p130) in Lmna-/- cells to determine whether or not the genome instability, telomere shortening, and altered nuclear positioning can be corrected by Rb expression. While such work might be outside the scope of the current manuscript, the authors should at least discuss this type of possibility.

6) In the text (page 13), the authors state that no chromosome end-to-end fusions were found in Lmna-/- MEFs, while the number of fusions presented in Suppl Fig 4C reflect that 4 fusions were identified. Therefore, the text should be modified accordingly. Albeit a low number, there is a two-fold increase in fusions (1.5 compared to 0.8) in Lmna-/- compared to Lmna+/+ cells. Is this significant? In addition, for comparison purposes, the results presented in Supplemental Fig. 4C regarding telomere fusions should be combined and presented in Fig. 5.

7) It would be nice if the authors could address whether or not there is a difference between Lmna-/- and Lmna+/+ cells in telomeric recombination as measured for example by telomeric sister-chromatid exchange (as performed previously by the authors in Gonzalo et al., Nat Cell Biol 8:416-424, 2006). In light of the increased genome instability (increased signal free ends, increased chromosome breaks, etc), such experiments would explain if or how cells lacking A-type lamins deal with these breaks. Furthermore it would be interesting for the authors to determine if the decrease in telomere fusions seen in Lmna-/- + TRF2 B M correlates with an increase in telomere recombination.

8) Although the authors demonstrate that Lmna+/+ and Lmna-/- cells were indeed transfected with a dnTRF2 (Fig 5B), they do not provide evidence for telomere uncapping and activation of a DNA damage response. Immunofluorescence experiments should be performed with antibodies against TRF1 and H2AX as well as TRF1 and 53BP1 (see below) to ensure that the dnTRF2 in Lmna-/- cells does indeed result in telomere uncapping. In addition, as TRF2 and POT1 have been shown to prevent end-to-end fusions, are there any differences in the expression of TRF2 and POT1 in Lmna-/- compared to Lmna+/+ cells?

9) In Fig 6, the authors demonstrate reduced 53BP1 expression in cells deficient or down-regulated for A-type lamins. In Fig 6A, the expression levels of 53BP1 are reduced in Lmna-/- cells while in Fig. 6B no 53BP1 protein is detected in these same cells. The authors should explain this discrepancy or show more than one film exposure. Additionally, the authors should perform immunofluorescence experiments (described above in point 8) as an alternative approach to look at 53BP1 expression and localization. Furthermore the authors should address whether the reduced protein levels of 53BP1 in the absence of A-type lamins is related to protein degradation by performing experiments in the absence and presence of proteasome inhibitors, or additional experiments to address how 53BP1 is destabilized. Finally, in Fig 6A, the authors fail to examine protein levels of several key NHEJ and DDR factors including XRCC4, DNA Ligase IV, DNA PKcs, XLF, and MDC1. Such results are necessary to make a point regarding the specificity of 53BP1 reduction.

10) Most importantly, the data presented in Figs. 5 and 6 merely show a correlation between 53BP1 expression and decreased telomere fusions in Lmna-/- + TRF2 B M cells. The authors need to provide a direct mechanistic link, for example, by repeating these experiments -/+ complementation

with 53BP1 as has been described previously (Dimitrova et al., Nature 456:524-528, 2008).

Minor comments:

a) Page 8, text. The range of mean telomere length for Lmna-/- MEFs should be changed to 34.0 to 35.2 kb in accordance with the table presented in Fig. 2B.

b) As the Tudor domain of 53BP1 has been reported to bind di-methylated H3K79 or H4K20, it would be interesting to examine these marks by ChIP using telomeric and pericentric probes as described in Fig 3.

Additional Correspondence from Authors to Editor 31 March 2009

Thanks for considering our manuscript for publication in EMBO Journal. We are very happy about the referees finding our observations of importance to understand molecular mechanisms altered upon loss of A-type lamins function which can be related to the pathophysiology of disease. We appreciate the suggestions offered by referee #3 to continue characterizing how A-type lamins participate in the regulation of telomere biology and the regulation of 53BP1 stability. We are planning to answer experimentally the questions raised by this referee. We are however, worried about the time that all the additional experiments might take us. In addition, we would like to know whether we need to take into consideration the comments of referee #2, once his/her comments are received.

Additional Correspondence from Editor to Authors 01 April 2009

Thank you for your email. I am sorry for the slight delay in getting back to you. First of all, I have not heard anything from referee #2 and at this stage we will just move ahead with the two reports that we have. So in other words there will be no third report. Regarding the concerns raised by referee #3. I find the comments provided by this referee very helpful and it will be important to address those concerns. I also recognize that it will be a lot of work to carry out the additional experiments, but what I can offer is that I can easily grant an extension up to 6 months if that would be helpful.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - authors' response 23 May 2009

Answers to Reviewer #2:

This is well-written manuscript that makes an important connection in the study of nuclear organization. The authors report connections between A-type lamin function and telomere biology. They find that cells lacking A-type lamins have altered distribution of mouse telomeres and altered heterochromatic marks at telomeres. Some of these findings can be linked to reduced levels of retinoblastoma protein family members, a known consequence of loss of A-type lamins. Others are independent of Rb family members. These findings are important since mutations in A-type lamins are linked to Hutchinson-Gilford progeria and altered telomere function is a possible link to disease mechanism. Also, loss of A-type lamin expression is linked to tumor progression and altered telomere function may be related to this phenotype as well. Of particular interest is the finding that

53BP1 levels are reduced in cells lacking A-type lamins. This could provide an explanation for altered DNA damage responses associated with laminopathies.

The data presented in this study is generally very sound. These findings will be of considerable interest to a wide readership and will foster further studies. There is one nominal concern (mostly editorial) that, if addressed, would further clarify the manuscript. Differences in the TRF analysis of adult fibroblasts (vs. MEFs) are stated to indicate that telomere defects are exacerbated during aging. This at least in the formal since is an overstatement. The authors should be clear what they mean by the term "aging," since these two populations of fibroblasts may be different for a range of reasons other than normal aging. In addition, telomere length appears to be longer in adult fibroblasts than MEFs, although cross-comparison may be difficult. Is there a reason for this?

We appreciate the reviewer's comments about the importance of our findings to understand molecular mechanisms behind alterations of A-type lamins function leading to disease states such as progeria (HGPS) or tumorigenesis. We also appreciate that the reviewer finds our data sound and of considerable interest to a wide readership. We agree with the reviewer that relating the exacerbated telomere shortening in adult fibroblasts upon loss of A-type lamins with aging is an overstatement. We have modified the text accordingly, and now we simply state that differences in telomere length are also observed in adult fibroblasts. We also agree with the reviewer in that telomeres in normal adult fibroblasts are longer than in wild-type MEFs. One difference to point out is that the analysis of telomere length in MEFs was performed prior to senescence and immortalization, while the analysis of telomere length in adult fibroblasts was performed after senescence and spontaneous immortalization. Immortalization of adult fibroblasts might contribute to a reset of telomere length homeostasis. We have observed changes in telomere length also when MEFs spontaneously immortalize, although we never investigated the molecular mechanisms behind this effect.

Answers to Reviewer #3:

In this manuscript, the authors investigate the role of A-type lamins on telomere biology and demonstrate that loss of A-type lamins results in altered distribution of telomeres in the nucleus, telomere shortening, reduced tri-methylation of H4K20 at telomeres, and increased genome instability associated with an increase in chromosome number and breaks. Furthermore, uncapping of telomeres in the absence of A-type lamins results in a reduction of telomere fusions (compared to Lmna+/+ cells), which the authors correlate with decreased protein levels of 53BP1. Overall, the manuscript is well written and the work is clearly presented. Furthermore, the data provide evidence for a number of interesting findings linking telomere biology to nuclear organization, and specifically the nuclear lamina. However, the data raise a number of questions and lack several key experiments as outlined below. In light of these issues, I feel that some further work would be needed before I could consider this work suitable for publication in EMBO J.

We appreciate the comments of the reviewer about the clarity of the manuscript, and the interesting nature of our findings linking telomere biology to nuclear organization. We have addressed experimentally the concerns raised by the reviewer.

1) The authors reference several publications demonstrating that cell cycle phase and senescence result in altered telomere positioning within the nucleus. As lamins are known to affect DNA transactions including replication, it is important to show cell cycle profiles (FACS analysis) of Lmna+/+ and Lmna-/- cells to determine whether or not cell cycle differences might contribute to the results presented in Fig 1. Along these same lines, since lamins are important for the stability of Rb, could differences in telomere distribution be related to differences in the percentage of senescent cells between Lmna-/- and Lmna+/+ cells? Experiments examining -gal staining (for example) would address this question.

We agree with the reviewer in that changes in the nuclear distribution of telomeres take place during the cell cycle, as well as during senescence. In particular, telomeres are distributed throughout the nucleoplasm in G0/G1/S phases of the cell cycle, while assembling into a telomeric

disk in the center of the nucleus during G2 (Chuang, Moshir et al. 2004). To date, a preferential localization of telomeres towards the nuclear periphery has not been reported at any stage of the cell cycle. Accordingly, no such pattern of telomere distribution was observed in asynchronously growing wild-type (Lmna+/+) MEFs. However, as suggested by the reviewer, we have performed FACS analysis to compare cell cycle profiles between Lmna+/+ and Lmna-/- MEFs (supplementary figure 1). These studies do not show differences in cell cycle profiles between these genotypes.

In addition, the 3D positioning of telomeres is altered in senescent cells presenting defects in the nuclear lamina (Raz, Vermolen et al. 2008). In particular, a preferred peripheral distribution of telomeres was observed in senescent cells. As suggested by the reviewer, an increase in the percentage of senescent cells could explain the changes in telomere distribution towards the nuclear periphery in Lmna-/- MEFs. However, the studies of telomere compartmentalization were performed in MEFs that had overcome senescence and spontaneously immortalize. We have clarified this point in the new version of the manuscript.

In summary, the changes in nuclear distribution of telomeres upon loss of A-type lamins are not due to differences in cell cycle profiles or number of senescent cells between the two genotypes.

2) In Fig. 1B and C, the authors find that loss of A-type lamins result in a shift in the localization of telomeres towards the nuclear periphery. In addition, using ChIP experiments they demonstrate that lamins A and C associate with telomeric DNA suggesting that lamins tether telomeres to the nuclear matrix. Wouldn't one expect that if lamins are important for tethering telomeres, that their loss would result in the movement of telomeres to the interior of the nucleus (away from the periphery)?

We completely agree with the reviewer that the opposite result was originally expected. Thus, we were very surprised by our findings that loss of A-type lamins leads to a shift in the distribution of telomeres towards the nuclear periphery. However, after performing the nuclear distribution studies many times, we are very confident that this is the case. We would like to direct the reviewer to the following paragraph in the discussion where this issue is addressed. "The change in localization of telomeres away from the nuclear center and towards the periphery upon loss of A-type lamins is intriguing. A-type lamins are highly enriched at the nuclear periphery, and are also found throughout the nucleoplasm (Schermelleh, Carlton et al. 2008). We reasoned that loss of A-type lamins could lead to detachment of telomeres from the nuclear periphery. Conversely, we found that the localization of telomeres shifts towards the nuclear periphery in the absence of A-type lamins, raising the possibility that the nuclear periphery represents a default pathway for telomere localization. In this model, A-type lamins would play an active role in the localization of telomeres throughout the entire nucleoplasm in mouse cells. A recent study showing that alterations of the nuclear lamina during senescence are associated with increased aggregation of telomeres at the nuclear periphery supports this model (Raz et al., 2008)".

3) A decrease in tri-methylation of H4K20 is demonstrated in Lmna-/- cells compared to wild-type (Fig. 3). Is this decrease due to reduced expression of SUV4-20H1 or SUV4-20H2? In addition, cells deficient for SUV4-20H showed reduced levels of H4K20me3 at telomeres, and these changes were accompanied by telomere elongation (Benetti et al., J Cell Biol 178:925-936, 2007). A mechanism (or at least a discussion) needs to be provided as to why reduced H4K20me3 correlates with longer telomeres as described by Benetti et al., and shorter telomeres described in this report.

The study by Benetti et al. mentioned by the reviewer as well as the study by Gonzalo et al.(Gonzalo, Garcia-Cao et al. 2005; Benetti, Gonzalo et al. 2007), demonstrated that the coordinated action of Suv4-20h1 and h2 HMTases and Rb family members is responsible for the stabilization of H4K20me3 at telomeres. Thus, Suv4-20h- or Rb family-deficiency are clear candidates for the decrease in H4K20me3 observed at telomeres of Lmna-/- MEFs. We decided to focus our attention on Rb family deficiency due to the fact that loss of A-type lamins had been previously shown to reduce considerably the levels of Rb, p107 and p130 (Johnson, Nitta et al. 2004). We confirmed the reduction in Rb family levels in our lines of Lmna-/- MEFs and concluded that the decrease in H4K20me3 is due at least in part to decreased levels of these proteins. We cannot rule out however, that decreased levels of Suv4-20h HMTases upon loss of A-type lamins could also contribute to the

decrease in H4K20me3 levels, and thus we discuss this possibility in the new version of the manuscript.

In addition, we have modified the text to make clearer the point that Rb deficiency and reduced H4K20me3 cannot explain the telomere shortening phenotype observed in Lmna-/- MEFs. We now discuss the possibility that A-type lamins play an active role in telomere elongation in the context of Rb and H4K20me3 deficiency.

4) It is not clear why the authors use immunoprecipitation followed by Western blotting to demonstrate loss or reduction of Rb, p130, and p107 in Lmna-/- cells compared to Lmna+/+ cells. Such experiments don't allow for loading controls. It
would be better just to prepare lysates (ex. in Laemmli buffer) and then probe for the levels of these proteins by Western blotting. These same lysates could be used to examine levels of SUV4-20H1 or SUV4-20H2, as mentioned above.

We originally monitored the levels of Rb, p107 and p130 in Lmna+/+ and Lmna-/- MEFs by immunoblotting total cell lysates. However, we were unable to detect any of these proteins by direct western. Thus, we modified the protocol and performed immunoprecipitation followed by western blot. Briefly, 1 ml of total cell lysate from Lmna+/+, Lmna-/-, and Rb-/-p107-/-p130-/- (TKO) MEFs -concentration 1 mg/ml- was subjected to immunoprecipitation with antibodies recognizing Rb, p107, and p130, or IgG as control. By this method, we were able to detect the levels of all three members of the Rb family. Most importantly, we obtained the same results as Johnson et al., who performed direct western blots in the same cells (Johnson, Nitta et al. 2004). The levels of Suv4-20h1 and h2 in mouse embryonic fibroblasts cannot be monitored by western blot due to the fact that antibodies that work in this assay are not available. However, we have modified the text to discuss the possibility that the levels of these proteins could be affected by the loss of A-type lamins.

5) Increased genome instability is demonstrated for Lmna-/- cells in Fig. 4 and Suppl Fig. 4. Several of these phenotypes, as well as the reduced H4K20me3 levels, are similar to cells lacking Rb family members. It would be interesting to complement and/or overexpress Rb (and/or other family members p107 and p130) in Lmna-/- cells to determine whether or not the genome instability, telomere shortening, and altered nuclear positioning can be corrected by Rb expression. While such work might be outside the scope of the current manuscript, the authors should at least discuss this type of possibility.

The experiments proposed by the reviewer are very interesting. However, we agree with the reviewer in that they are outside the scope of the current manuscript. We now discuss in different parts of the manuscript the phenotypes observed in Lmna-/- MEFs that could be due to the decrease in Rb family function. In particular, the changes in H4K20me3 upon loss of A-type lamins are likely to be due to decreased Rb family function. However, the telomere shortening phenotype, the decrease in TERRAs levels and the increase in telomere loss (signal free ends) cannot be explained by Rb deficiency. We propose that A-type lamins participate in the regulation of different aspects of telomere biology via Rb-dependent and Rb-independent mechanisms.

6) In the text (page 13), the authors state that no chromosome end-to-end fusions were found in Lmna-/- MEFs, while the number of fusions presented in Suppl Fig 4C reflect that 4 fusions were identified. Therefore, the text should be modified accordingly. Albeit a low number, there is a two-fold increase in fusions (1.5 compared to 0.8) in Lmna-/- compared to Lmna+/+ cells. Is this significant? In addition, for comparison purposes, the results presented in Supplemental Fig. 4C regarding telomere fusions should be combined and presented in Fig. 5.

We have modified the text so that the presence of some fusions in Lmna+/+ and Lmna-/- MEFs is stated. Performing a t-test of statistical significance we find that the differences are not significant, and are due probably to stochastic telomere dysfunction in both genotypes. As suggested by the reviewer we have included the data of telomere fusions prior to telomere dysfunction by expression of TRF2 B M in the table in new figure 5C.

7) It would be nice if the authors could address whether or not there is a difference between Lmna-/- and Lmna+/+ cells in telomeric recombination as measured for example by telomeric sister-chromatid exchange (as performed previously by the authors in Gonzalo et al., Nat Cell Biol 8:416-424, 2006). In light of the increased genome instability (increased signal free ends, increased chromosome breaks, etc), such experiments would explain if or how cells lacking A-type lamins deal with these breaks. Furthermore it would be interesting for the authors to determine if the decrease in telomere fusions seen in Lmna-/- + TRF2 B M correlates with an increase in telomere recombination.

As suggested by the reviewer, we have performed CO-FISH to determine whether the loss of A-type lamins leads to increased levels of telomere recombination. New supplementary figure 6 shows that depletion of A-type lamins does not lead to an increase in the frequency of recombination events involving telomeres. Therefore, aberrant telomere recombination cannot explain the defects in telomere metabolism and the increase in signal free ends and genomic instability observed upon loss of A-type lamins.

8) Although the authors demonstrate that Lmna+/+ and Lmna-/- cells were indeed transfected with a dnTRF2 (Fig 5B), they do not provide evidence for telomere uncapping and activation of a DNA damage response. Immunofluorescence experiments should be performed with antibodies against TRF1 and H2AX as well as TRF1 and 53BP1 (see below) to ensure that the dnTRF2 in Lmna-/- cells does indeed result in telomere uncapping. In addition, as TRF2 and POT1 have been shown to prevent end-to-end fusions, are there any differences in the expression of TRF2 and POT1 in Lmna-/- compared to Lmna+/+ cells?

As suggested by the reviewers we have performed Immuno-FISH to determine if expression of TRF2 B M induces telomere uncapping and activation of the DNA damage response pathway in Lmna+/+ and Lmna-/- MEFs (new figure 5B). Our results show that the expression of TRF2 B M leads to the formation of TIFs both in Lmna+/+ and Lmna-/- MEFs.

In addition, we have performed western blots with TRF1, TRF2, and POT1 antibodies (new figure 6D). No differences in the levels of these proteins were observed between Lmna+/+ and Lmna-/- MEFs, indicating that alternative mechanisms are responsible for the deficiency in NHEJ of dysfunctional telomeres.

9) In Fig 6, the authors demonstrate reduced 53BP1 expression in cells deficient or down-regulated for A-type lamins. In Fig 6A, the expression levels of 53BP1 are reduced in Lmna-/- cells while in Fig. 6B no 53BP1 protein is detected in these same cells. The authors should explain this discrepancy or show more than one film exposure. Additionally, the authors should perform immunofluorescence experiments (described above in point 8) as an alternative approach to look at 53BP1 expression and localization. Furthermore the authors should address whether the reduced protein levels of 53BP1 in the absence of A-type lamins is related to protein degradation by performing experiments in the absence and presence of proteasome inhibitors, or additional experiments to address how 53BP1 is destabilized. Finally, in Fig 6A, the authors fail to examine protein levels of several key NHEJ and DDR factors including XRCC4, DNA Ligase IV, DNA PKcs, XLF, and MDC1. Such results are necessary to make a point regarding the specificity of 53BP1 reduction.

We have modified figure 6 to clarify the discrepancy of the previous figure, which was the result of different exposure of the western blot. We now show that depletion of A-type lamins by shLmna leads to a decrease in the levels of 53BP1. Most importantly, reintroduction of lamins A, C, or both into A-type lamins depleted cells leads to the recovery of 53BP1 levels (new figure 6E). These new results clearly demonstrate that A-type lamins play a key role in the regulation of 53BP1 levels.

In addition, and following the reviewers advice, we have performed immunofluorescence assays with 53BP1 antibody. As shown in figure 6B, when pictures were taken under the same exposure conditions, a clear decrease in the intensity of labeling with 53BP1 antibody was observed upon

loss of A-type lamins. These new experiments confirm the results obtained previously by immunoblotting.

Furthermore, we have performed western blots in the presence of proteasome inhibitors (new figure 6G). Briefly, cells growing in culture were treated with cycloheximide to inhibit protein synthesis and with cycloheximide and MG132, an inhibitor of the proteasome. We found that cycloheximide treatment for 6 hours markedly reduces the cellular levels of 53BP1. However, the combined treatment with cycloheximide and MG132 leads to a pronounced increase in the levels of 53BP1 protein. These results indicate that 53BP1 protein degradation involves the proteasome pathway. We discuss in the new version of the manuscript the possibility that A-type lamins might participate in the stabilization of 53BP1 by binding to the protein, and preventing its degradation by the proteasome, such is the case of Rb family members (Johnson, Nitta et al. 2004).

Finally, we have evaluated how the loss of A-type lamins impacts on the levels of additional factors participating in the DDR and DNA repair. In particular, we have monitored the levels of MDC1, ATM, DNA-PK, and ERCC1 (new figure 6C). We found no detectable changes in the levels of these proteins in Lmna-/- MEFs, suggesting that A-type lamins stabilize preferentially 53BP1 levels. Nevertheless, we have modified the text to make very clear that other factors involved in NHEJ could be affected by the loss of A-type lamins, contributing to the phenotype.

10) Most importantly, the data presented in Figs. 5 and 6 merely show a correlation between 53BP1 expression and decreased telomere fusions in Lmna-/- + TRF2 B M cells. The authors need to provide a direct mechanistic link, for example, by repeating these experiments -/+ complementation with 53BP1 as has been described previously (Dimitrova et al., Nature 456:524-528, 2008).

As suggested by the reviewer we attempted to reintroduce 53BP1 into Lmna-/- MEFs and rescue NHEJ of dysfunctional telomeres. However, we encountered different technical difficulties, which are shown in the supplementary figures included for the reviewer. As shown in figure 1A retroviral transduction of Lmna-/- MEFs with 53BP1 construct markedly increased the levels of 53BP1. Subsequenlty, we introduced the TRF2 MB construct via retroviral transduction with two different vectors pLPCpuro and pWZLhygro. As shown in figure 1B, expression of TRF2 MB varied tremendously in the different cell lines, hindering our ability to compare the levels of chromosome end-to-end fusions in 53BP1 proficient and deficient Lmna-/- lines. We tried to overcome these difficulties by lentivirally transducing 53BP1 expressing Lmna-/- MEFs with shRNAs specific for depletion of TRF2. As shown in figure 1C, two out of five shRNAs decreased the levels of TRF2 mRNAs to approximately 30%. However, this decrease was not sufficient to induce chromosome end-to-end fusions.
These difficulties prevent us from testing at this time whether 53BP1 loss is solely responsible for the inhibition of NHEJ upon loss of A-type lamins. Thus, we have modified the text to clearly state that loss of A-type lamins leads to decreased levels of 53BP1, and that the notion that this decrease of 53BP1 hinders NHEJ is speculative. Other strategies will need to be developed to address the fundamental question of how A-type lamins regulate 53BP1 protein stability. We would like to stress however, that this question is not to the main focus of our manuscript. The main message of our study is the demonstration that loss of A-type lamins leads to alterations of the nuclear distribution of telomeres that are accompanied by defects in telomere structure, length and function. In addition, our study establishes that A-type lamins are necessary for the processing of dysfunctional telomeres by NHEJ, and for the stabilization of 53BP1, a key component of the DNA damage response pathway.

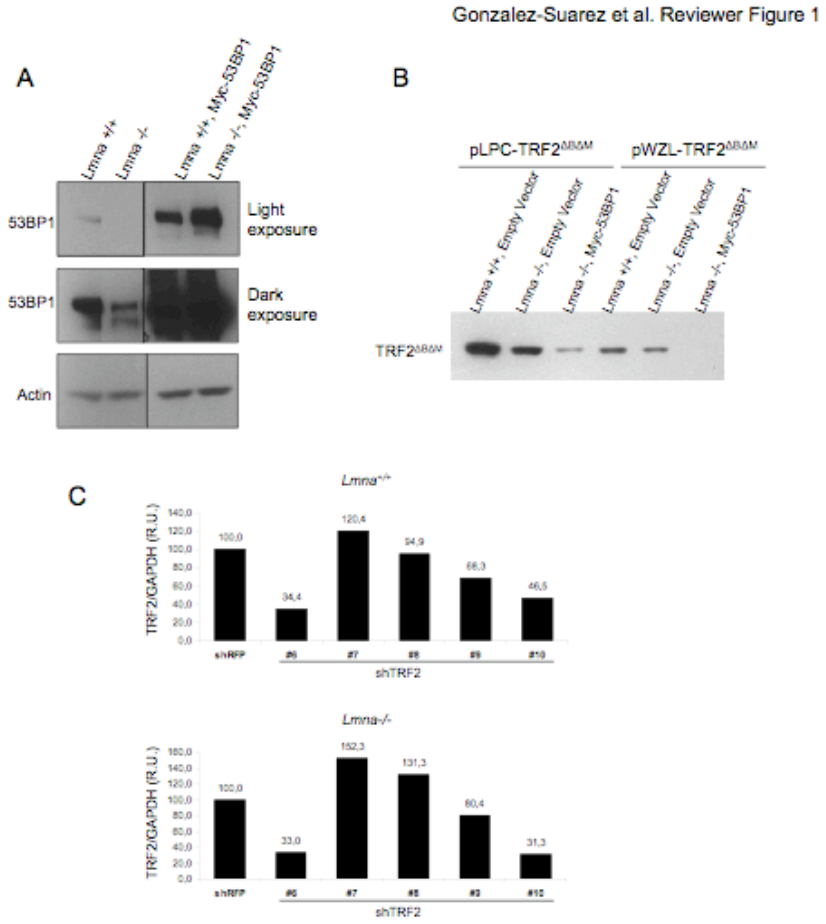
Minor comments:

a) Page 8, text. The range of mean telomere length for Lmna-/- MEFs should be changed to 34.0 to 35.2 kb in accordance with the table presented in Fig. 2B.

We thank the reviewer for point out to us this mistake.

b) As the Tudor domain of 53BP1 has been reported to bind di-methylated H3K79 or H4K20, it would be interesting to examine these marks by ChIP using telomeric and pericentric probes as described in Fig 3.

As suggested by the reviewer, we have monitored the global levels of H3K79me2 and H4K20me2 in Lmna+/+ and Lmna-/- MEFs. The results are included as supplementary figure 2 for the referee. As shown in the figure, no differences in the levels of H4K20me2 and a modest decrease in H3K79me2 levels were observed upon loss of A-type lamins. These results, together with the data showing that 53BP1 is targeted to degradation via the proteasome pathway, will be the basis of future studies to elucidate the molecular mechanisms by which A-type lamins regulate 53BP1 protein stability.

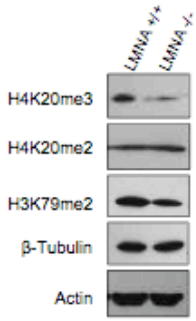


A. Reconstitution of 53BP1 levels in Lmna-/- MEFs. Western blots with 53BP1 antibody after retroviral transduction of Lmna+/+ and Lmna-/- MEFs with a Myc-53BP1 construct. Note the high levels of expression of 53BP1, specially in Lmna-/- MEFs.

B. Expression of TRF2$^{\Delta B\Delta M}$ in 53BP1 deficient and proficient Lmna MEFs. Lmna+/+ and Lmna-/- MEFs retrovirally transduced with an empty vector or with Myc-53BP1 were selected and subsequently transduced with either pLPC- TRF2$^{\Delta B\Delta M}$ or with pWZL-TRF2 $^{\Delta B\Delta M}$ to induce telomere dysfunction. Western blots with TRF2 antibody show that different lines express different levels of the dominant negative protein. Therefore, the extent of telomere dysfunction and chromosome end-to-end fusions cannot be compared between cell lines deficient and proficient in 53BP1.

C. Depletion of TRF2 by shRNAs. Lmna+/+ and Lmna-/- MEFs were lentivirally transduced with five shRNAs targeted to TRF2. Note how shRNAs numbers 6 and 10 depleted the TRF2 transcripts to approximately 30% of shRFP control. However, no telomere dysfunction in the form of chromosome end-to-end fusions were observed up to seven days in culture. The remaining TRF2 might be sufficient to maintain telomere function.

Gonzalez-Suarez et al. Reviewer Figure 2



Changes in histone modifications upon loss of A-type lamins. Western blots comparing the levels of three post-translational modifications of histones in Lmna+/+ and Lmna-/- MEFs. In addition to the described decrease in H4K20me3, we found some decrease in H3K79me2 levels. In contrast, no changes in H4K20me2 were observed upon loss of A-type lamins.

2nd Editorial Decision 03 June 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #3 to review the revised manuscript and I have now received the comments. As you can see below, this referee finds that you have satisfactorily addressed the original issues raised and supports publication here. However, the referee also has a few remaining issues that need to be resolved before formal acceptance here. I would therefore like to ask you to respond to the remaining points raised by referee #3 in a final round of revision. When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #3 (Remarks to the Author):

The authors have gone a long way towards addressing the various issues I raised in my previous review, and the manuscript is consequently significantly improved on the original. Overall, I feel that this work is suitable for acceptance in EMBO Journal. However, I still have three issues that I feel that the authors should address before this work is formally accepted:

ï In the revised figure Suppl. 6, the authors use shRNA depleted cells. This analysis apparently could also have been carried out in MEFs, which were used in various other assays. Could the authors re-do this experiment with MEFs? If not, can they explain why?

ï A formal possibility for the data in Fig. 5 A/B is that uncapping did not occur effectively in the -/- cells. Previous data in the manuscript showed that -/- cells have elevated basal gamma-H2AX. To demonstrate uncapping in the -/- cells, the authors should analyze gamma-H2AX by western immunoblotting (before and after TRF2delta induction).

ï To make a clear conclusion from the experiment for Fig.6g, the samples really need to be analyzed on the same gel.

2nd Revision - authors' response 12 June 2009

Answers to Reviewer #3:

The authors have gone a long way towards addressing the various issues I raised in my previous review, and the manuscript is consequently significantly improved on the original. Overall, I feel that this work is suitable for acceptance in EMBO Journal. However, I still have three issues that I feel that the authors should address before this work is formally accepted:

We appreciate the nice comments of the reviewer, and have now addressed experimentally the three remaining concerns raised.

In the revised figure Suppl. 6, the authors use shRNA depleted cells. This analysis apparently could also have been carried out in MEFs, which were used in various other assays. Could the authors re-do this experiment with MEFs? If not, can they explain why?

As suggested by the reviewer, we have performed CO-FISH analysis in immortalized Lmna+/+ and Lmna-/- MEFs. As shown in new Supplementary figure 6, no differences in the frequency of recombination involving telomeric sequences were observed upon loss of A-type lamins.

A formal possibility for the data in Fig. 5 A/B is that uncapping did not occur effectively in the -/- cells. Previous data in the manuscript showed that -/- cells have elevated basal gamma-H2AX. To demonstrate uncapping in the -/- cells, the authors should analyze gamma-H2AX by western immunoblotting (before and after TRF2delta induction).

As suggested, we have performed two different assays to monitor the uncapping of telomeres upon expression of TRF2 B M in Lmna+/+ and Lmna-/- MEFs.
First, we performed immunofluorescence studies to quantitate the percentage of cells presenting more than 5 foci of DNA damage labeled with H2AX upon retroviral transduction with empty vector or TRF2 B M. New Supplementary figure 7A shows similar percentage of cells presenting DNA damage upon expression of TRF2 B M in Lmna+/+ and Lmna-/- MEFs.
Second, western blot analysis shows a marked increase in H2AX levels upon expression of TRF2 B M in Lmna+/+ and Lmna-/- MEFs when compared to an empty vector control. New Supplementary

figure 7B.

To make a clear conclusion from the experiment for Fig.6g, the samples really need to be analyzed on the same gel.

We have performed the experiment once more, and loaded all the samples into the same gel. We agree with the reviewer in that it clarifies the conclusion.
We would like to express our most sincere gratitude to the reviewer for helping us to improve the quality of the manuscript.

