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Organismal propagation in the absence of a functional telomerase pathway in Caenorhabditis elegans

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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 07 December 2011

We have now received all three reports on your C. elegans ALT paper, which I am sending you copied below in this email. As you will see, these reports are somewhat ambivalent: referee 1 is in principle supportive of publication pending clarification of a few specific issues, while referee 3 is not convinced that your present data have lead to decisive new insights suitable for a broad major journal. Finally, referee 2 considers the establishment of a metazoan model of long-term survival without telomerase in principle very interesting, but raises some doubts whether your present set of data really support the notion of this survival reflecting a bona fide 'worm ALT' as opposed to some other protective mechanism; s/he further provides several concrete experimental suggestions that may help to clarify this question.

With these mixed recommendations at hand, I feel the overall interest expressed by two of the three reviewers would justify considering a revised version of the manuscript further for publication. For such a revision to be successful, it will however be essential to address the concerns raised by referees 1 and especially 2, which on the whole all aim towards a better understanding of the molecular basis of telomere protection in the survivors. Regarding the criticisms of referee 3, I do not agree with their somewhat dismissive sentiment, but would also like you to carefully consider and respond to their concerns regarding causalities and possible alternative explanations for survival/propagation without telomerase.

Should further experimentation along the lines requested by referees 1 and 2 allow you to substantiate the evidence for a bona fide ALT mechanism operating in the double mutant worm survivors, we shall be happy to consider the study for eventual acceptance. In this respect, please note that we will not consider competing papers that may appear while your manuscript is under revision as compromising novelty, meaning that you should be able to fully exploit our usual three months revision time frame to address the main concerns. At the same time, I should however also stress that I am not able to predict the referees' support for a revised manuscript should the requested

experiment fail to support a bona fide ALT and instead point to some other mechanism of alternative telomere maintenance or survival; in such situation, eventual acceptance would depend on whether or not at least referees 1 and 2 would still consider such a different protective mechanism of sufficient interest by itself.

Given the well-taken criticisms and the competitive situation, it may be helpful for you to get back to me with any responses/concerns you may have regarding the referee comments at any stage prior to resubmission of a revised manuscript; furthermore I would appreciate if you could keep us updated with any news you may have on the status of the competing work you mentioned.

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

With best regards,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In the manuscript entitled 'Organismal propagation in the absence of a functional telomerase pathway in Caenorhabditis elegans', Lackner and colleagues describe the generation of worms that survive for up to 180 generations without the telomerase enzyme. To date, trt1-1 mutant worms have not been shown to activate telomerase-independent means of telomere maintenance and eventual telomere erosion leads to organismal sterility. The authors use a ceob2 mutant strain background as a tool to generate trt1-1 mutant worms that can propagate indefinitely. The authors demonstrate that ceob2 mutant strains exhibit an increased level of c-circles, which are thought to be an indicator of increased telomeric recombination. They demonstrate that the deletion of trt1 in ceob2 mutant strains allows the isolation of trt1-1/ceob2 worms that maintain heterogeneous telomeres, reminiscent of the ALT phenotype seen in mammalian cells. The authors claim that trt1-1/ceob2 mutant worms suffer telomeric fusions and genome instability. They also profile the global gene expression pattern of a trt1-1/ceob2 mutant clone and draw comparisons with trt1-1 and ceob2 single mutant worms. Overall, this is an interesting and straightforward study and the generation of ALT animals provides a valuable tool for studying ALT at both a mechanistic and an organismal level.

The paper can be improved by addressing the following issues:

1) The text would benefit from a more in-depth description of c-circles, including the c-circle assay and a clearer explanation of the relationship between c-circles and ALT.
2) In figure 4, it is interesting that the high molecular weight material does not hybridize intensely with the telomere probe, as does the slower migrating material in single mutant ceob2 samples. This could suggest that fusion occurred specifically between chromosome ends without many telomere repeats, or it could suggest something like cerevisiae type I where subtelomeric recombination dominates and only small stumps of actual telomeric repeats are left on each chromosome end. Also the much fainter telomere hybridization in trt1-1 ceob2 compared to ceob2 suggests a tight interplay between telomerase activity and recombination (or whatever ceob2 controls). It could warrant more discussion.
3) The data in figure 5 do not provide convincing evidence for telomere fusions. The Bal31 assay used previously (Raices et al, 2008) should be used to determine whether the slow migrating bands in figure 4 contain fusions. An RNAi approach against lig-4 to show a rescue of the putative fusion phenotype (if the fusions are NHEJ-dependent) would also be informative.
4) In all figures that contain Southern blots, the probe used should be indicated underneath the figure

for clarity.
5) In Figure 1A-C, it would be useful to have the wells of the gel clearly indicated. In Figure 1C, the quantity of M13 ssDNA shown in the gel label is inconsistent with what is stated in the Figure legend (pg versus ng).
6) In figure 5: Unclear what the 4 horizontal panels represent. Also unclear which cell types are captured, as the text refers to oocytes at diakinesis and the legend refers to them as mitotic cells in the gonad, please clarify.
7) The quantitation in figure 5b shows a bias for either 5 or 1-3 chromosome masses. To clarify it would be interesting to define which chromosomes are connected/fused by using a FISH approach to reveal which chromosomes are fused (co-localization foci of X-chromosome and chromosome I for example). Published FISH probes for specific chromosome arms like rDNA (chromosome V), chromosome I or an X-chromosome repeat (as in Phillips et a., NCB 2009 and Smolikov et al. PLoS Genet 2008) could also be used.
8) Figure 6a plots the expression profile of a gene localized close to the telomere, the authors should include proof that the gene is actually intact and present in the ALT worm line double mutant trt-1 ceob2 (B; F110) and was not lost due to the recombination in telomere regions. Also, it would be important to assess the expression profiles of multiple ALT clones to find a consensus between survivors. Finally, the expression profile does not show many differences except for one gene (clec-190). It would be helpful if the authors would describe what is known about this gene and what its role could be in these ALT worms.

Referee #2 (Remarks to the Author):

This manuscript describes the generation of strains of C. elegans that lack both telomerase and a telomere-binding protein, yet maintain their telomeres sufficiently to survive for many generations. To my knowledge it is the first description of long-term whole-organism "survivors" lacking telomerase in multicellular organisms, and hence is of great interest to the telomere field.

However, I am not convinced by the conclusion that this phenomenon is the worm version of ALT, the "alternative lengthening of telomeres" mechanism that has been described in immortal human cells, for the following reasons:
1) The most specific marker of human ALT yet described is the C-circle assay, which was used in this study to demonstrate an ALT-like phenotype in the parental strain lacking CeOB2. For some reason the double mutant survivors were not also tested with the C-circle assay; a positive result in this assay would greatly increase confidence that the phenomenon is ALT-like.

2) By definition, ALT involves a lengthening of telomeres, but it is not clear from Figure 4 that there has been any telomere lengthening in the C. elegans double mutants. The overall amount of telomeric DNA hybridizing to the probe is approximately the same as or less than the wt parental strain, and certainly much less than the CeOB2 mutant parental strain.

3) The authors classify the telomere phenotype in the double mutants as "heterogeneous", but it looks like most of the telomeres are no more heterogeneous in length than the wt strain (for example, the majority of telomeric DNA in the far right lane of Figure 4B is in a smear with a mean length shorter than that in the wt strain), and certainly do not display the ALT-like pattern seen in the CeOB2 single mutants. There are higher molecular weight bands that come and go over the generations, but the authors postulate that some of these are due to telomeric DNA at chromosomal fusions. It seems possible that all of these bands represent fused telomeres. This could be addressed by carrying out a Bal31 digestion of the DNA prior to probing for telomeric DNA.

4) While the survivors do survive for at least 180 worm generations, they seem to be barely holding on - about 90% of the progeny at every generation have become sterile. If their behaviour was ALT-like, you would expect the population to go through a crisis until ALT was activated, and offspring of that fertile animal would then take over the population.

Could it be instead that the survivors are managing to survive due to the protection of a portion of their telomeres in fused chromosomes? This would be more similar to the telomerase-negative S. pombe strains that survive by circularising their chromosomes, than to the recombination-based mechanism of human ALT. It would also be consistent with the similarities in gene expression

profile found in the double mutants and the late-generation telomerase mutants. In this regard, it would be interesting to see how the pattern of chromosome fusions changed in each clone over time, and whether there is a correlation between the number of remaining unprotected telomeres and the propagation ability of the clone.

Referee #3 (Remarks to the Author):

This manuscript describes the isolation of C. elegans strains that can be maintained for many generations without telomerase activity, as such a novel finding. Such strains, which have a reduced number of chromosomes (most likely the result of chromosomal fusions), were obtained in ceob2 mutant backgrounds.

The key observation in this manuscript may be the result of a more broad repertoire of viable karyotypes in C. elegans, and to me it is unclear whether ceob2 functionality is a requirement to prevent these: the strains that were obtained and can be perpetuated have altered numbers of chromosomes leading to the question whether strains with altered chromosome number (e.g. carrying the meT7 rearrangement) that were derived in an alternative way now also can produce progeny in the absence of telomerase.

In my opinion the current manuscript, while presenting a interesting feature of C. elegans biology, is highly descriptive and does not presenting sufficient novel (mechanistic) insight in telomere protection mechanisms nor detailed insight into the structural changes of the altered strains and whether and how these depend on ceob2.

I don't have comments on technical aspects nor on the textual content of the manuscript as it was a nice read.

1st Revision - authors' response 01 February 2012

We would like to thank the referees for their constructive criticism, which has made this a much better manuscript. Below we have addressed all points in detail. For clarity we have kept the referee's points in bold and our responses in regular script. Where applicable, we have pointed out page and lines that have been altered in the manuscript.

Referee #1 (Remarks to the Author):
In the manuscript entitled 'Organismal propagation in the absence of a functional telomerase pathway in Caenorhabditis elegans', Lackner and colleagues describe the generation of worms that survive for up to 180 generations without the telomerase enzyme. To date, trt1-1 mutant worms have not been shown to activate telomerase-independent means of telomere maintenance and eventual telomere erosion leads to organismal sterility. The authors use a ceob2 mutant strain background as a tool to generate trt1-1 mutant worms that can propagate indefinitely. The authors demonstrate that ceob2 mutant strains exhibit an increased level of c-circles, which are thought to be an indicator of increased telomeric recombination. They demonstrate that the deletion of trt1 in ceob2 mutant strains allows the isolation of trt1-1/ceob2 worms that maintain heterogeneous telomeres, reminiscent of the ALT phenotype seen in mammalian cells. The authors claim that trt1-1/ceob2 mutant worms suffer telomeric fusions and genome instability. They also profile the global gene expression pattern of a trt1-1/ceob2 mutant clone and draw comparisons with trt1-1 and ceob2 single mutant worms. Overall, this is an interesting and straightforward study and the generation of ALT animals provides a valuable tool for studying ALT at both a mechanistic and an organismal level.

Response:
We thank the referee for the positive remarks about the manuscript.

Referee #1:
The paper can be improved by addressing the following issues:
1) The text would benefit from a more in-depth description of c-circles, including the c-circle assay and a clearer explanation of the relationship between c-circles and ALT.

Response:
We have included a more detailed discussion of c-circles in the revision (page 6, lines 7-14).

Referee #1:
2) In figure 4, it is interesting that the high molecular weight material does not hybridize intensely with the telomere probe, as does the slower migrating material in single mutant ceob2 samples. This could suggest that fusion occurred specifically between chromosome ends without many telomere repeats, or it could suggest something like cerevisiae type I where subtelomeric recombination dominates and only small stumps of actual telomeric repeats are left on each chromosome end.
Also the much fainter telomere hybridization in trt1-1 ceob2 compared to ceob2 suggests a tight interplay between telomerase activity and recombination (or whatever ceob2 controls). It could warrant more discussion.

Response:
We thank the referee for pointing this out. We find it difficult to draw definite conclusions from the telomere length analysis of the CeOB2 mutants and comparison to the double mutants, since the telomeric signal is so strong in the CeOB2 -/- animals. It is well possible that fusions form preferentially at very short telomeres, and we have briefly addressed this now in the text (page 11, last 5 lines; page 12, lines 1-2). We would however like to point out that it could be rather misleading to compare the strong signal from long telomeres that are beyond the resolution capacities of the gels to the multiple bands in the double mutants.

Referee #1:
3) The data in figure 5 do not provide convincing evidence for telomere fusions. The Bal31 assay used previously (Raices et al, 2008) should be used to determine whether the slow migrating bands in figure 4 contain fusions. An RNAi approach against lig-4 to show a rescue of the putative fusion phenotype (if the fusions are NHEJ-dependent) would also be informative.

Response:
We have now included Bal-31 digestions (Figure 5C), which demonstrate the presence of internal telomeric repeats due to fusions and these results are discussed on page 11, lines 5-17.
We did not manage to perform the proposed lig-4 experiment, as the lig-4 RNAi treatment would have to be done in the initial stages of the crosses and would therefore be extremely time consuming and out of the time frame of a revision. Furthermore, it is unclear how the animals would react to long-term suppression of the NHEJ pathway and lig-4 seems to be dispensable for telomeric fusions in C. *elegans* (Lowden et al, Genetics 180, 741-754, 2008).

Referee #1:
4) In all figures that contain Southern blots, the probe used should be indicated underneath the figure for clarity.

Response:
We thank the referee for pointing this out and we have included the used probes in all the figures.

Referee #1:
5) In Figure 1A-C, it would be useful to have the wells of the gel clearly indicated. In Figure 1C, the quantity of M13 ssDNA shown in the gel label is inconsistent with what is stated in the Figure legend (pg versus ng).

Response:
We thank the referee for pointing out the problems and we have now indicated the wells as requested and corrected the error in the legend of Figure 1C.

Referee #1:
6) In figure 5: Unclear what the 4 horizontal panels represent. Also unclear which cell types are captured, as the text refers to oocytes at diakinesis and the legend refers to them as mitotic cells in the gonad, please clarify.

Response:
We thank the referee for pointing out the problems and we have now clarified the cell types and panels in the figure legend.

Referee #1:
7) The quantitation in figure 5b shows a bias for either 5 or 1-3 chromosome masses. To clarify it would be interesting to define which chromosomes are connected/fused by using a FISH approach to reveal which chromosomes are fused (co-localization foci of X-chromosome and chromosome I for example). Published FISH probes for specific chromosome arms like rDNA (chromosome V), chromosome I or an X-chromosome repeat (as in Phillips et a., NCB 2009 and Smolikov et al. PLoS Genet 2008) could also be used.

Response:
We fully agree with the referee that this would be an extremely informative and rewarding approach. However, there are technical limitations that we could not overcome within the time frame of resubmission of the revision. First, we have experimented with FISH analysis of mitotic chromosomes and found that the protocols for meiotic chromosomes are not appropriate. We are in the process of troubleshooting our attempts and of developing protocols for mitotic FISH, however we expect this to take several more months.
Second, to draw absolute conclusions we would need specific subtelomeric probes for at least half of the chromosomes. While the subtelomeric regions in nematodes are relatively unique with fewer repetitive sequences than mammalian chromosomes, it is still quite an undertaking to identify all these probes. Again, we expect this to take several months.
We therefore hope that the referee agrees with us that, while a wonderful suggestion, we could not complete these experiments yet.

Referee #1:
8) Figure 6a plots the expression profile of a gene localized close to the telomere, the authors should include proof that the gene is actually intact and present in the ALT worm line double mutant trt-1 ceob2 (B; F110)
and was not lost due to the recombination in telomere regions.

Response:
We have confirmed the presence of the cTel55X.1 gene in the double mutants by PCR on genomic DNA using two different primer sets in two independent lines (DM B, DM D). The results have now been indicated in the text (page 13, lines 18-20) and are shown as Supplemental Figure 3.

Referee #1:
Also, it would be important to assess the expression profiles of multiple ALT clones to find a consensus between survivors.

Response:
As requested, we have profiled two more double mutant lines (DM C and DM D), and the trend for the regulation of genes is very similar to that shown in the original manuscript. We have added the data as Supplemental Figure 4, which shows the clusters of genes regulated in the DM B according to the gene expression values of DM C and DM D. Also, there are similar enriched gene-ontology terms, which have been added to Supplemental Table 2.

The results have been discussed in the text at the end of the Results section (page 15, line 9 to page 16, line 4).

Referee #1:
Finally, the expression profile does not show many differences except for one gene (clec-190). It would be helpful if the authors would describe what is known about this gene and what its role could be in these ALT worms.

Response:
We do not think that this gene plays a role in ALT, as it is also differentially expressed between the two wild-type controls and probably has a more variable gene expression pattern in general. We have added this to the text (page 14, lines 15-17).

We would like to thank the referees for their constructive criticism, which has made this a much better manuscript. Below we have addressed all points in detail. For clarity we have kept the referee's points in bold and our responses in regular script. Where applicable, we have pointed out page and lines that have been altered in the manuscript.

Referee #2 (Remarks to the Author):

This manuscript describes the generation of strains of C. elegans that lack both telomerase and a telomere-binding protein, yet maintain their telomeres sufficiently to survive for many generations. To my knowledge it is the first description of long-term whole-organism "survivors" lacking telomerase in multicellular organisms, and hence is of great interest to the telomere field.

However, I am not convinced by the conclusion that this phenomenon is the worm version of ALT, the "alternative lengthening of telomeres" mechanism that has been described in immortal human cells, for the following reasons:

Response:
We deeply thank the referee for the generous comments about the interest of our manuscript to the field. We have addressed the referee's concerns in detail, as outlined below.
While our worm strains might not exactly mimic what has been observed in immortal human cell lines without telomerase, we would like to point out that there is a wide variety in the human lines as well. Many ALT lines have long and heterogeneous telomeres, but not all. Saos2 and KMST6 have relatively short telomeres, despite the lack of telomerase activity (Oganesian et al., Mol. Cell 42, 224-236, 2011). This points out that a lot of heterogeneity exists even between human ALT lines.
Since ALT is generally referred to as pathways that allow telomere maintenance in the absence of telomerase, I would politely suggest that not all characteristics have to apply to all ALT pathways .

Referee #2:
1) The most specific marker of human ALT yet described is the C-circle assay, which was used in this study to demonstrate an ALT-like phenotype in the parental strain lacking CeOB2. For some reason the double mutant survivors were not also tested with the C-circle assay; a positive result in this assay would greatly increase confidence that the phenomenon is ALT-like.

Response:
We thank the referee for pointing this out and fully agree. We focused on the CeOB2 mutants because we thought it was important to show that lack of CeOB2 was indeed related to an ALT-like phenotype. We have now added the c-circle assay for two independent survivor lines, and the assay points out an increase in c-circles in both. The data is now shown in Figure 4A/B and described in the text on page 9, lines 2 to 5.

Referee #2:
2) By definition, ALT involves a lengthening of telomeres, but it is not clear from Figure 4 that there has been any telomere lengthening in the C. elegans double mutants. The overall amount

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of telomeric DNA hybridizing to the probe is approximately the same as or less than the wt parental strain, and certainly much less than the CeOB2 mutant parental strain.

Response:
I would politely suggest that the most importance characteristic of ALT is the presence of telomere length maintenance in the absence of telomerase activity. This is certainly happening in the animals here. We fully agree with the referee that absolute telomere length seems to be variable (as it is for human ALT cell lines).
We find it difficult to draw definite conclusions from the telomere length analysis of the CeOB2 mutants and comparison to the double mutants, since the telomeric signal is so strong in the CeOB2 -/- animals. I would like to point out that it could be rather misleading to compare the strong signal from long CeOB2 telomeres that are beyond the resolution capacities of the gels to the multiple bands in the double mutants.
We have now performed Bal-31 digests as suggested by the referee, and this analysis points at the presence of long telomeres in the double mutants (please see below).

Referee #2:
3) The authors classify the telomere phenotype in the double mutants as "heterogeneous", but it looks like most of the telomeres are no more heterogeneous in length than the wt strain (for example, the majority of telomeric DNA in the far right lane of Figure 4B is in a smear with a mean length shorter than that in the wt strain), and certainly do not display the ALT-like pattern seen in the CeOB2 single mutants. There are higher molecular weight bands that come and go over the generations, but the authors postulate that some of these are due to telomeric DNA at chromosomal fusions. It seems possible that all of these bands represent fused telomeres. This could be addressed by carrying out a Bal31 digestion of the DNA prior to probing for telomeric DNA.

Response:
We have now done the Bal-31 digests and they are now shown as Figure 5c. The results suggest the presence of telomeric fusions, and the presence of long telomeres in the double mutants (especially DM D), consistent with the ALT phenotype. This is described in the manuscript on page 11, lines 5-17.

Referee #2:
4) While the survivors do survive for at least 180 worm generations, they seem to be barely holding on - about 90% of the progeny at every generation have become sterile. If their behaviour was ALT-like, you would expect the population to go through a crisis until ALT was activated, and offspring of that fertile animal would then take over the population.

Response:
This is a very important point, but I would like to politely suggest that it is a misunderstanding. What we were trying to show in Figure 2D was that 90% of the animals don't survive, but once they became survivors, they have decent brood sizes (color coded green and blue), as defined by Ahmed et al, Nature 403, 159-164, 2000. We apologize for the confusion and have attempted to clarify this in the text now (page 7, line 19). Also, the strains are becoming healthier with increasing generations, however we would prefer not to go into details here, since the phenomenon is difficult to quantify accurately.

Referee #2:
Could it be instead that the survivors are managing to survive due to the protection of a portion of their telomeres in fused chromosomes? This would be more similar to the telomerase-negative S. pombe strains that survive by circularising their chromosomes, than to the recombination-based mechanism of human ALT. It would also be consistent with the similarities in gene expression profile found in the double mutants and the late-generation telomerase mutants. In this regard, it would be interesting to see how the pattern of chromosome fusions changed in each clone over time, and whether there is a correlation between the number of remaining unprotected telomeres and the propagation ability of the clone.

Response:
We appreciate the suggestions of the referee, however we are faced with technical limitations to explore the exact nature of the fusions in the survivor strains, preferentially by FISH analysis with chromosome specific probes. First, we have experimented with FISH analysis of mitotic chromosomes and found that the protocols for meiotic chromosomes are not appropriate. We are in the process of troubleshooting our attempts and of developing protocols for mitotic FISH, however I expect this to take several more months.
Second, to draw absolute conclusions we would need specific subtelomeric probes for at least half of the chromosomes. While the subtelomeric regions in nematodes are relatively unique with fewer repetitive sequences than mammalian chromosomes, it is still quite an undertaking to identify all these probes. Again, I expect this to take many months.
I therefore hope that the referee agrees with us that, while a wonderful suggestion, we could not complete these experiments yet.
We do not find indication for circularized chromosomes at this point, based on the lack of ring-chromosomes in the analysis presented as Figure 5A, the Bal-31 digests and the moderate offspring in the double mutants that argues against meiotic problems.

Referee #3 (Remarks to the Author):

This manuscript describes the isolation of C. elegans strains that can be maintained for many generations without telomerase activity, as such a novel finding. Such strains, which have a reduced number of chromosomes (most likely the result of chromosomal fusions), were obtained in ceob2 mutant backgrounds.

The key observation in this manuscript may be the result of a more broad repertoire of viable karyotypes in C. elegans, and to me it is unclear whether ceob2 functionality is a requirement to prevent these: the strains that were obtained and can be perpetuated have altered numbers of chromosomes leading to the question whether strains with altered chromosome number (e.g. carrying the meT7 rearrangement) that were derived in an alternative way now also can produce progeny in the absence of telomerase.

In my opinion the current manuscript, while presenting a interesting feature of C. elegans biology, is highly descriptive and does not presenting sufficient novel (mechanistic) insight in telomere protection mechanisms nor detailed insight into the structural changes of the altered strains and whether and how these depend on ceob2.

I don't have comments on technical aspects nor on the textual content of the manuscript as it was a nice read.

Response:
We thank the referee for positively commenting on the readability of the manuscript.

We do not think it is likely that strains with altered chromosome number generally allow for survival without telomerase. Our laboratory and Shawn Ahmed's laboratory have extensively searched for *ad hoc* survivors emerging from telomerase negative strains but despite frequent changes in chromosome numbers in such animals we could never identify one. It is therefore likely that survivors depend on a driver for telomeric recombination, such as the CeOB2 deletion.

We respectfully disagree about the importance of the manuscript, since we maintain that the strains described here represent the first multicellular model for ALT in an organism with canonical telomeres. While we do not fully understand the mechanism of ALT activation at this point, we suggest that CeOB2 is a regulator of telomeric recombination and that these strains will be useful tools to screen for other ALT regulators.

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Acceptance letter 13 February 2012

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees 1 and 2, and I am pleased to inform you that both of them are satisfied with the revision and have no more objections towards publication in The EMBO Journal.

Referee 2 has one more comment (see below) regarding the clarity of one particular figure legend - I will leave it to your discretion whether or not to make any minor changes in response. Please briefly let me know, and (in case) simply attach a modified text file for us.

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

With best regards,

Editor
The EMBO Journal

Referee #1

(Remarks to the Author)

Referee #2

(Remarks to the Author)
I was reviewer #2 of the original manuscript. In their revisions and additional experiments, the authors have addressed all of my concerns very effectively. In particular, the extra data demonstrating that the survivors have increased levels of C-circles, as well as long telomeres that are not fused, reassures me that the mechanism of their survival probably involves telomeric recombination.

Regarding my point 4: I get it now - the "% surviving clones" in each column refers to the % of the original clones, whereas I was interpreting it as the % of offspring from the generation before (i.e. I thought that the survivors from generation 160 in the left panel produced offspring that were 90% sterile). Maybe to make this even clearer, so the reader doesn't make the same mistake I did, you could change the wording in the figure legend to "percentage of the original clones still surviving" rather than "percentage of surviving clones".