Deletion of Ogg1 DNA glycosylase results in telomere base damage and length alteration in yeast

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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 03 April 2009

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by three experts in the field and their comments to the authors are provided below. As you can see, the referees find the analysis potentially interesting, however they also indicate that the analysis would require substantial revisions in order for further consideration here. In general, the referees are not convinced that, at this stage, that the analysis provides conclusive enough data in support for the main findings reported. Better quantification is needed and some of the findings need to better controlled and extended. I realize that the referees suggest a significant amount of further experimental work, but the analysis would have to be extended along these lines in order for publication here. It is not clear if the concerns raised can be fully resolved, but if you are able to do so, then we would be willing to look at a revised version. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

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

Editor
The EMBO Journal

REFEREE REPORTS
In this manuscript, Lu and Liu present the results of a study on the influence of guanine oxidation on telomere dynamics. There are several central findings. First, DNA damage is produced at the telomere in vivo. Second, the ogg1 null allele gives rise to Pif1-independent longer telomeres that is dependent on both telomerase, as expected, and, to a lesser extent, by recombination. This is true both of Y′ class telomeres and individual telomeres. Second, there is some growth synergy between telomerase-negative Rad52-negative cells resulting in an increased rate of senescence. Third, the binding of the key telomere size regulator Rap1 is reduced in ogg1 mutant cells suggesting that lack of Rap1 negative regulation gives rise to elongated telomeres. Fourth, ogg1 has a mild influence on telomere position effects.

This study provides the first evidence for an effect of oxidized G at telomeres in vivo and for their biological effect. The Rap1 connection provides a meaningful mechanistic model that is based on the high G-rich content of Rap1 consensus site. The studies are well controlled and of high quality. Overall, these results provide an intriguing connection between DNA damage and telomere function that may be relevant to aging. The silencing result is less important since the cause of the phenotype (size or gene product) remains unknown.

However, there is a concern regarding the identity of the est2 strain. Figure 4. Lane 3 (and Figure 7A) shows an est2 strain that has a pre-senescent phenotype without accumulating Type I or Type II survivors. This would indicate that the strain never passed through senescence after 115 generations of subculturing, an odd finding inconsistent with est2 solid subculturing in Figure 7A that shows death without survivors after around 100 generations. This is surprising and requires comment. The concern here is the identity of the est2 strain.

Referee #2 (Remarks to the Author):

In this manuscript, the authors provide evidence that oxidized guanines at telomeric repeats affect telomere homeostasis. This is based on their central finding that ogg1 cells accumulate oxidized guanines at telomeric TG1-3 repeats compared to wild-type cells. This result in telomere lengthening, which they claim is dependent on the telomerase and recombination pathways. In my opinion, however, the results show that this increase is most likely independent of the telomerase pathway. Importantly, the authors also convincingly show that the binding of a key component of telomere homeostasis regulation, Rap1, to the telomeric TG1-3 repeats is highly sensitive to the presence of oxidized guanines in vitro and that this is a possible cause for the mild alteration of Rap1 binding seen in vivo.

These are potentially very interesting findings. There are, however, substantial problems within the current manuscript (see below). Contradictory results are not discussed (telomerase dependence is a striking example) and there is an overall lack of convincing statistical analysis of the data.

1. The evidence that ogg1 cells exhibit a higher level of 8oxoG at telomeres compared to that in wild-type cells is poorly documented and not convincing (Fig 1):
   - In Fig 1B, a positive control has to be included (oxidative treatment on wild-type cells) and most importantly the ogg1 mutant complemented with pRS316-OGG1. This should be easy as the strains and constructs are available (Fig 3A) and will support the central claim that increased levels of 8oxoG lead to telomere lengthening.
   - It is not clear (even from the reference given in the material and method) how quantification was achieved. I would like to read directly in the result section how the quantitation were done, particularly the normalization between ogg1 untreated and ogg1 treated was calculated. The signals used for quantification should be marked on Fig 1B.

2. That OGG1 deletion leads to telomere lengthening isn't new, see Askree SH et al. (2004) PNAS. This initial discovery has to be mentioned in the result section! Instead, the novelty consists in the quantitation of the increased telomere length and in the observation of dispersed telomere length in ogg1 cells. While the quantitation needs better presentation and analysis, the dispersal of telomere length is only discussed and needs better description and presentation in the result section.
   - Fig 2A, what is the error for the quantitation? Is the graph representing the mean of 3 independent
experiments (it should)? Please also comment in the text/discussion on the length instability of the long non-telomeric (?) TG1-3 repeat containing sequences (see also point 5). In Fig 2B, I would like to see a quantification as in Fig 2A with statistics. More importantly, the increased spreading of telomere length has to be quantified as well.

- Before using ogg1 strains to study the effect of oxidized guanines on telomere length homeostasis (p7 top), the authors should first make the hypothesis that ogg1 doesn’t affect telomere homeostasis through an indirect pathway. This has also to be discussed more extensively later on (contribution of the mitochondrial Ogg1 for instance).

- The exclusion of the contribution of the nearby gene Pif1 to the telomere phenotype of ogg1 cells is nicely done, but to the fact that this possibility was already proposed in Askree et al. (2004), so please refer to it. Fig 3C however needs quantitation since the corresponding statement (p10 top) about the independency from Pif1 has important consequences (see point 3 below).

3. The claim that ogg1 altered telomere length homeostasis depends on both telomerase and recombination pathway is not supported by the data (Fig 4). On Fig 4A, I see a similar increase (if anything can be seen) in ogg1 cells in the wild-type or in the est2 background. Therefore, I would conclude that Est2 and Ogg1 act independently. This would then be consistent with the fact that ogg1 effect is independent of the catalytic inhibition of the telomerase by Pif1, according to the result on Fig 3C. The current contradiction between the claimed results of Fig 4A and those of Fig 3C must anyway be discussed if this result is solid.

- We need to be provided with sound quantitative data for the epistasis analysis with Est2 and Rad52 presented on Fig 4A and B. This is critical since epistasis would be borderline, and wrong assignment would lead to opposite conclusions.

4. The in silico analysis of Rap1 binding to 8oxoG is flawed as the authors do not make clear distinction between their previous published results and their own extrapolation (p11). Overall Fig 5 isn’t much informative and could be reduced to one panel (D?) or dropped. Color of dashed lines are hardly distinguishable.

5. The authors claim that Rap1 binding to telomeric repeats is inhibited by 8oxoGs. In vitro data are convincing, and should be compared with similar studies like the mammalian TRF1/TRF2 binding at oxidized telomeric sequences (Opresko et al. (2005) NAR. For the in vivo part, I am concerned by the ChIP and TPE (telomere positioning effect) data. Since Rap1 is binding to other sequences than telomeric DNA in the genome (5% of the promoters) and has additional function than that at telomeres, the results should be more cautiously presented. For the ChIP, the observed decreased Rap1 occupancy at TG1-3 is likely to reflect binding to non telomeric TG1-3 sequences as these are probably immunoprecipitated as well (bound by Rap1) and represent the highest part of detectable TG1-3 repeats as shown Fig 2A. This should also be discussed in relation with the size of the sonicated DNA fragments and the effect of increased telomeric length in ogg1. Why not use Rif1 or Rif2, as they are telomere specific and mutants are viable? As the TPE effect is borderline and likely to be indirect, it doesn't deserve its own result section.

7. Last but not least, there is a general lack of consistency and discussion throughout the manuscript and especially in the discussion section. Obvious contradictions (see above) are not indicated nor discussed.

Conclusion:
The manuscript needs substantial revision and some additional experiments. However, provided the above concerns are addressed, I think the novelty and originality of the results deserve publication in the EMBO Journal.

Referee #3 (Remarks to the Author):

The authors have analyzed telomeric DNA damage and length in S. cerevisiae lacking the glycosylase Ogg1 compared to wild type. Ogg1 acts in repair of oxidized guanine bases. The authors show depletion of Ogg1 leads to a slight increase in oxidized guanines in the telomeres and telomere elongation that they argue depends on telomerase and recombination. Oxidized guanines led to disruption of Rap1 protein binding to telomeric DNA in vitro, and Ogg1 deletion led to decreased association of Rap1 with telomeres in cells. The authors conclude that oxidized guanines at the
telomeres inhibit Rap1 binding and increase access to telomerase and recombination proteins which lengthen the telomeres. The manuscript is generally well written and attempts to determine the mechanism of telomere elongation. However, the data fall short of supporting the conclusions and proposed mechanisms.

Major comments:
The detection of 8oxoG lesions by the assay in Fig. 1 is not readily apparent. One would expect an obvious shift to faster migrating fragments as observed for UV dimers in the referenced manuscript (Kruk et al 1995) that used the same method. Such a shift is not obvious in this manuscript for oxidative lesions. A positive control (H2O2 treatment) would help confirm that the assay is working properly. It would be most helpful if the method for quantitating the lesions was included (at least in the supplemental materials). Is there evidence that ogg1 deletion yeast strains contain more 8oxoG in the genome in general? (this should be cited). It is not surprising that the telomeres would also sustain increased 8oxoG in the absence of ogg1. The authors point out that previous work showed telomere DNA is susceptible to damage in cells, and that telomeric sequence in vitro is highly prone to oxidative damage (reviewed in Passos et al NAR, 2007 and von Zglinicki Trends Biochem Sci, 2002). Have the authors compared the level of 8oxoG damage in telomeres to genomic DNA?; this would be highly interesting.

The authors document an increase in telomere length comparing generation 40 to 65 in the ogg1 deletion strain, but no further increase in subsequent generations (115 and 215). How do the authors explain this? Shouldn't damage continue to accumulate in the telomeres in the absence of ogg1 with each generation? Based on their model, more damage should lead to less Rap1 binding and increased telomerase and Rad52 access and thus, more lengthening (Fig. 9). In Fig. 2A it appears that the smears representing the Y' telomeres shift up with each generation in both the wt and ogg1 deletion strains. Wouldn't this suggest telomere lengthening with generation in both strains? The authors do not see telomere lengthening in the ogg1 negative strain for the XVL telomeres; except for one, although the shift is barely detectable. Do these telomeres contain less damage? Also the authors report in the introduction that the (TG(1-3)) repeats are 256-375 bp (pg. 3), but show lengths of 1200 to 1400 bp in Fig. 2A.

A great number of previous reports in mammalian cells indicate that oxidative damage induces telomere shortening (reviewed in Passos et al NAR, 2007 and von Zglinicki, Trends Biochem Sci, 2002). The current study suggests that yeast differ from mammalian cells in this regard. This is a very important point for discussion. Have the authors found that oxidative damage (i.e. H2O2 treatment) also triggers telomere lengthening in yeast?

The authors have not convincingly shown that the telomere lengthening detected in the ogg1 deletion strain depends on recombination, or even Rad52. The smear representing Y' telomeres in the ogg1 and rad52 double deletion strain appears to migrate the same as the telomere smear from the ogg1 single deletion strain at generation 115 (Fig. 4B). The comparison is hard to make for generations 40, 64, and 90 (Fig. 7B) since the ogg1 single deletion strain is not shown on the same gel as the double ogg1 rad52 deletion strain, but based on the markers they appear very similar length. It os very difficult to interpret slight differences in migration of the Y' telomere smears at the bottom of the gel. Quantitation and demonstration of reproducibility among several independent spores might help. Is there any evidence from the literature that telomeres are lengthened by Rad52 dependent recombination in the presence of telomerase activity? Why wouldn't telomerase be able to contribute to more extensive telomere lengthening in the rad52 ogg1 double deletion mutant, since it can in the ogg1 single mutant? The data in Fig. 4A that show telomerase is required for telomere lengthening in the ogg1 negative strain is much more convincing.

Overlapping standard deviations for the TPE effect between the wild type and ogg1 deletion strains suggest that there is no effect; 9+/-3 compared to 15+/-6 and 21+/-5 compared to 30+/-9.

The authors tested Rap1 binding to a duplex that has two Rap1 binding sites. However, the binding is not significantly decreased unless both Rap1 binding sites have an 8oxoG. Based on the previously demonstrated sequence specify of Rap1 binding, it is not surprising that altering the sequence would alter affinity. At a lesion frequency of 65 per 10<sup>6</sup> bases (as reported by the authors), how likely is it that two lesions would be in adjacent binding sites? Since the lesions are rare it also seems unlikely that Rap1 binding would be greatly impacted, contrary to what is depicted in the model in Fig. 9. The data from the ChiP assay are compelling and suggest that Rap1
association with telomeres is decreased in the ogg1 deletion strain, but the mechanism is far from clarified. One would expect a much higher frequency of 8oxoG lesions in the telomeres if these adducts were responsible for the decreased Rap1 association. Is Rap1 expressed at similar levels in the strains? Are there similar levels of Rap1 in the immunoprecipitates from both strains?

The interpretation of the cell streaks in Fig 7 is not clear. The est2 (telomerase) deletion strain shows a progressive decrease in cell growth from streak 1 to 4. But the est2 and ogg1 double deletion strain shows similar growth in streaks 1 and 2, an increase in growth in streak 3, and then a dramatic loss of growth in streak 4. One expects a progressive decline in survival from streak 2 to 4. How do the authors explain the variability? How reproducible is this "wave" effect in the double mutant? A more quantitative analysis, such as standard liquid senescence assays or spotting of serial dilutions of cultures (starting with the same number of cells) would greatly clarify any differences between the single and double mutants. Data showing that the strains exhibit similar growth rates is important to include. The result that the ogg1 est2 rad52 triple mutant showed rapid loss of viability is not that interesting (contrary to what the authors claim, pg. 14), since the same result was observed with the est2 rad52 double mutant (which is not novel). Ogg1 did not alter the phenotype.

The authors claim that (pg. 14) the double ogg1 est2 double mutant showed mild telomere lengthening compared to the est2 single mutant. This is not observed in the gels in Fig. 7B or the graph. The brown (triangle) and blue (x) lines are almost perfectly aligned. Error bars are certainly required. Furthermore, the 700 bp fragment in the gels in 7B shift up, showing a brown in the gel. This makes it very difficult to detect a slight shift in the Y' telomere smears. In this gel the telomeres from the wild type and ogg1 negative strains appear very similar, contrary to what is shown in the graph.

Minor Comments
In the discussion the authors' reference to "individual" telomeres is confusing (pg. 16). The telomeres are all analyzed in bulk population. Analysis of individual telomeres requires STELA or qFISH on chromosome spreads.

1st Revision - authors' response 02 October 2009

Responses to the reviewers' comments:

We appreciate the reviewer’s critical comments on our manuscript and incisive suggestions for improvement. We have addressed the reviewer's concerns point by point as follows:

Reviewer #1 was generally satisfied with the manuscript in its content. We appreciate the reviewer’s critical comments on the identity of est2 deletion strain. This strain, in our hands, had a similar senescent phenotype as described by others, because it had greatly reduced colonies after a few streaks and the appearance of survivors in the subsequent streaks. In the original figure, est2 deletion mutant appeared to be viable after 3 streaks from the colonies formed from tetrad analysis. Past ~100 generations after spore germination, est2 mutant cells had started to display heterogeneous colony morphology, a sign of onset of senescence. We agree with the reviewer that the original figure did not clearly present the appearance of survivors. To better clarify the difference among streaks and the mutants, we included results of a spotting assay of serial dilutions of liquid cultures at different passages (Figure 7). As expected, est2 deletion strain showed reduced spotting capability and subsequent survivors. We also agree with the reviewer that the silencing effect in ogg1Δ strain is in borderline. We included this statement in the revised manuscript (see page 16, the last paragraph).

Reviewer #2 also found that the manuscript represented interesting findings. In general, this reviewer felt a need for statistical analysis of the telomere length data and for discussion of controversial results regarding telomerase-dependent telomere lengthening in ogg1 mutant in the context of Pif1p function. We agree with the reviewer’s suggestions and have significantly strengthened the statistics with careful quantification and calculation. We also added the discussion
of the results from pif1 and ogg1 mutants in the revised manuscript accordingly and addressed the reviewer’s other comments point by point as follows:

Point 1: According to the reviewer’s suggestion, we included the positive controls (i.e. oxidative treatment in vitro and in vivo), and ogg1Δ mutant complemented with pRS316-OGG1 (Figure 1). We also documented in detail the quantification of base lesions in Figure S1 and material and methods. We showed that in vitro and in vivo H2O2 treatment could increase telomeric oxidative base lesions that were readily detectable by the method, and that the wild-type OGG1 gene complemented base damage phenotype of ogg1Δ mutant. Higher concentration of H2O2 also led to increased level of smaller telomere fragments without Fpg treatment, perhaps because it caused single strand breaks in telomeric DNA (Oikawa et al., FEBS Lett, 453, 365-368, 1999). Additionally, we have improved the measurement sensitivity by digesting DNA with 4 bp cutters to reduce the size of the telomere restriction fragments. Since the method does not detect the absolute number of Fpg-sensitive lesions in a sample, we presented fold-changes in each sample, which was calculated against the frequency of Fpg-sensitive lesions in the control. To the reviewer’s request, the signals used for quantification is illustrated in Figure S1.

Point 2: We cited Askree’s paper in the discussion section of our previous manuscript. In the revised manuscript, we referred to this paper twice in the result section (see page 7 and 10, the last paragraph).

We have added error bars for the telomere length quantatition, representing the mean of three independent experiments. The telomere length measurement was performed according to Askree’s paper that is cited in the material and method (see page 28, the second paragraph). In Figure 2A, the individual bands immediately above Y’-containing telomere fragment are non-Y-containing single telomeres, because these bands disappear during the formation of Type I survivors that maintain these telomeres by Y’-element amplification. The bands with size of 5.2kb and 6.7kb are Y’-elements (Lundblad and Blackburn, Cell 73:347-60, 1993). Unlike Y’-containing telomeres and non-Y’-containing telomeres, Y’-elements did not show amplification in ogg1Δ strain (Figure 2A). Figure 2B further confirmed that Y’-elements were not amplified in ogg1Δ strain.

The reviewer pointed out that Ogg1p was also known to repair oxidative base damage in mitochondrial DNA. To test if telomere lengthening is the byproduct of mitochondrial DNA damage, we deleted mitochondria DNA in wild type and ogg1Δ strains. Loss of mitochondrial DNA (rho0) in wild type and ogg1Δ strains did not affect their telomere length (Figure S4), which is consistent with a recent report showing that telomere length is unaltered in Saccharomyces cerevisiae with a rho0 background (Meng et al, EMBO J, 28:1466-78, 2009). Thus, telomere lengthening in ogg1Δ strain is independent of mitochondrial DNA damage.

For the Pif1 analysis, we cited Askree’s paper (see page 10, the last paragraph) and added quantitative measurement in Figure 3C accordingly.

Point 3: It is our fault that a non-representative ogg1Δ mutant was presented in Fig 4A of the previous version and caused the confusion. We have revised this figure. After vigorously testing different clones of est2 and ogg1 deletion mutants, we are confident that telomere lengthening in ogg1 deletion mutant depends on telomerase, as telomere length in ogg1Δ est2Δ clones is significantly shorter than that of ogg1Δ cells but similar to est2Δ cells (Figure 4A).

Pif1p is a helicase that removes telomerase from chromosome ends through unwinding of the telomeric DNA and telomerase RNA duplex, while Ogg1p can repair oxidative guanine lesions that would otherwise perturb telomere protein complex’s negative regulation upon telomerase (Boule et al, Nature, 438: 57-61, 2005; Nucleic Acids Res. 35: 5809-18, 2007; Zhang et al, Nucleic Acids Res. 34: 1393-404, 2006, and the current study). Thus, both Pif1p and Ogg1p appear to regulate telomere length through telomerase-mediated telomere elongation, but as mentioned above they act on telomerase by distinct mechanisms. This discussion was included in the revised manuscript (see page 20, the last paragraph).
To the reviewer’s request, the telomere lengths were quantified and SE values were added in Figure 4A and B. These data support our suggestion that ogg1Δ-mediated telomere lengthening may be dependent on telomerase and/or Rad52 pathways.

Point 4: We placed this figure as a supplementary figure (Figure S6) according to the reviewer’s suggestion. The structure of Rap1p bound to telomere DNA was reported by Dr. Daniela Rhodes’s laboratory previously (Konig et al., Cell, 85, 125-136, 1996). However, it is not known how 8-oxoG in the telomere DNA affects the binding of Rap1p. We thus conducted in silico structural analysis of Rap1p’s binding to telomeres containing 8-oxoG bases. We believe that this analysis will help clarify the mechanism on how 8-oxoG perturbs Rap1p’s function in telomeres.

Point 5: We showed that 8-oxoG in the Rap1p-binding sites adversely affects the binding of Rap1p to telomere DNA in vitro. This finding is consistent with previous in vitro observation by Opresko et al that the amount of 8-oxoG in telomere repeat sequences affects the binding of mammalian telomere binding proteins, TRF1 and TRF2 to the telomeric DNA (Nucleic Acids Res, 33, 1230-1239, 2005). To the reviewer’s request, we added it to the result section (see page 15, the second paragraph).

For the CHIP analysis, we sheared formaldehyde-cross-linked chromatin DNA to a standard size (500-1000 bp) in order to keep most telomeres intact. As pointed out by the reviewer, it is possible that some DNA signals detected by the telomere probe in the Rap1p CHIP analysis is attributable to TG1-3 repeats from intergenic regions. To further strengthen our observation that decreased Rap1p’s binding to DNA occurred in the telomeric region, we examined the association of Rap1p-interacting protein, Rif2p to telomeres in ogg1Δ strain by CHIP according to the reviewer’s suggestion. We found that like Rap1p, Rif2p’s binding to telomere DNA decreased in ogg1Δ strain (Figure 6B). Because Rif2 interacts with Rap1p at the telomeric site, a reduction of Rif2’s binding directly supports that oxidative guanine lesions in telomeres perturb the affinity of telomere protein complex to telomeres. We also explored the telomere lengthening phenotype of ogg1Δ mutant in rif1Δ rif2Δ background. The extreme length and heterogeneity of telomeres in rif1Δ rif2Δ mutant makes it impossible to determine if OGG1 deletion may lead to additional elongation (data not shown).

We agree with the reviewer that the TPE effect in ogg1Δ strain is in borderline. As suggested, we regrouped this section together with cell viability studies (see page 16, the last paragraph).

Point 7: To the reviewer’s constructive suggestion, we have reformatted results and discussion sections, in particular adding a new paragraph discussing Rif1p and Ogg1p in telomerase-mediated telomere lengthening (page 20, the last paragraph).

Reviewer #3 We have carefully addressed all the reviewer’s comments, and categorized the comments and answers as follows:

Major comments:
Detection of oxidative base lesions

According to the reviewer’s suggestion, we have included positive controls (H2O2 treatment in vitro and in vivo) in Figure 1 and the methods in quantification of base lesions in Figure S1 and material and methods. We have shown that telomeric oxidative base lesions were readily detectable after H2O2 treatment by the method employed here (Figure 1, B and C). Higher concentration of H2O2 also led to increased level of smaller telomere fragments without Fpg treatment, perhaps because it caused single strand breaks in telomeric DNA (Oikawa et al., FEBS Lett, 453, 365-368, 1999). Krük et al previously detected pyrimidine dimmers in telomeric DNA using the same assay (PNAS 92:258-262, 1993). They showed that pyrimidine dimmers only became readily detectable, when cells were exposed to extreme high dose of ultraviolet light. Similarly, we found that endogenous oxidative base damage was not readily detectable in telomeres in wild type cells. However, the level of oxidative base lesions increased in telomeres, when cells were challenged with a lethal dose of H2O2 briefly (Figure 1C). Ablation of Ogg1p function could also increase oxidative base lesions in telomeres (Figure 1D). Under these conditions, a slight shift towards smaller telomere DNA fragments was detected after Fpg treatment and was recognizable by the imaging software analysis.
Detection of 8-oxoG in the genome of ogg1Δ strain has not been reported previously in S. cerevisiae. As suggested by the reviewer, Fpg-sensitive lesions in the genomic DNA were measured by the method described in this study, and they were not significantly different in wild-type and ogg1Δ cells (see the above figure). It is possible that Fpg-sensitive lesions are relatively low in the genome of both wild type and ogg1Δ cells or are below the level of detection by the method. Although we have vigorously explored methods for detecting oxidative base lesions in single telomeres and genome-wide, we are unable to establish a reliable assay at this point and thus did not include the data in the revised manuscript.

![Genomic DNA from representative spores with indicated genotypes](image)

Telomere lengthening phenotype
We have shown that telomere length of ogg1Δ strain increased with progressive passages, reaching a plateau (370 to 407 bp) after ~65 generations (Figure 2A). This limited telomere lengthening may be because endogenous oxidative damage is limiting or a compensatory response is induced that sets base damage and repair to a new equilibrium. We therefore tested if increased oxidative stress would enhance the telomere lengthening phenotype in ogg1Δ strain (see detailed discussion below).

The increase band size in wild type cells was an artifact of gel smiling in the original Figure 2A. We now included a 700 bp telomere fragment as an internal migration control and showed that wild-type cells had a relatively steady telomere size in successive passages (Figure 2A). Due to the usage of XhoI restriction enzyme, telomere restriction fragments (TRFs) include Y’ elements that make “short telomere fragments” appear larger than their actual sizes by Southern blot analysis. For a consistent presentation of telomere length in the introduction and figures, we subtracted the 875 bp Y’ element from the value of TRFs in our calculations according to the published protocol (LeBel, C., et al, Assessing telomeric phenotypes. Methods Mol Biol, 313, 265-316. 2006) and have revised Figure 2 and manuscript (see the last paragraph of page 7, the second paragraph of page 28, and Figure 2 legend) accordingly.

Oxidative guanine damage is associated with telomere lengthening in S. cerevisiae. The reviewer pointed out that this observation is not in agreement with previous findings that oxidative stress leads to telomere shortening in mammalian cells (Thomas von Zglinicki, Ann N Y Acad Sci. 908: 99-110, 2000). However, given the fact that telomere lengthening was also observed in Ogg1 deficient mouse tissues and primary MEFs cultivated in low ambient oxygen (our unpublished data) and that the experiments in mammalian cells were conducted under oxidative stress condition that causes DNA strand breaks, it is possible that different types of oxidative DNA damage, e.g. DNA strand breakages or base lesions may have different impact on telomere length (either lengthening or shortening). We have added this statement in the discussion (see page 20, the second paragraph).
According to the reviewer’s suggestion, we tested if H$_2$O$_2$ treatment would enhance the telomere lengthening phenotype. Surprisingly, non-lethal doses of H$_2$O$_2$ do not lead to telomere length change in wild type and ogg1Δ strain (data not shown). A similar conclusion was reported by another lab in a recent Telomere and Telomerase meeting. It is noteworthy that non-lethal doses of H$_2$O$_2$ did not significantly increase the number of Fpg-sensitive lesions in ogg1Δ strain (data not shown); unless the cells were briefly treated with a lethal dose of H$_2$O$_2$ (see Figure 1C). We thus tested if an increase in endogenous H$_2$O$_2$ could enhance telomere lengthening in ogg1Δ strain. Tsa1p, one of peroxiredoxins, has the most potent ability to scavenge H$_2$O$_2$. To increase endogenous H$_2$O$_2$ in ogg1Δ strain, ogg1Δ tsa1Δ double mutant was generated. The tsa1Δ mutant had longer telomeres than the wild type, and the double mutant displayed longer telomeres than either single mutants (Figure S2). This result implies that increased endogenous oxidative stress can exacerbate telomere lengthening in the absence of Ogg1p.

**Telomerase and recombination in telomere lengthening**

As requested by the reviewer, we have repeated the experiments and performed quantitation on multiple independent ogg1 rad52 deletion clones. Our results confirm the Rad52p-dependent telomere lengthening in some ogg1 deletion clones (Figure 4B). This find was further supported by our new data showing that deletion of RAD50 diminishes telomere lengthening in ogg1Δ clones (Figure S6). In telomerase defective strain, two types of survived cells can emerge by maintaining telomeres via recombination. Type I survivors maintain telomeres by Y’ element amplification; while type II survivors have increased telomere repeats. Type I survivors depend on Rad51p and Rad52p, and Type II survivors depend on Rad50p and Rad52p, respectively. As shown in Figure S6, removal of RAD51 had no effect on telomere lengthening in ogg1Δ strain, while deletion of RAD50 completely diminishes telomere lengthening in some ogg1Δ clones. Thus, deletion of OGG1 can trigger telomere recombination that mimics type II survivors of telomerase defective mutants. These results further strengthen that recombination is involved in telomere lengthening of ogg1Δ strain.

It has been shown that deletion of the RAD52 gene does not affect telomere length maintenance in *S. cerevisiae* with intact telomerase. Rad52p is, however, essential in telomere length maintenance in the survivors of telomerase defective strains, after telomeres are exhausted and trigger cell death (Lundblad and Blackburn, *Cell*, 73, 347-360, 1993). Interestingly, Rad52p can mediate telomere lengthening in the presence of telomerase, when the OGG1 gene is deleted. We have discussed this in connection with possible mechanisms of Rad52p and Rad50p-dependent telomere lengthening in ogg1Δ strain in the revised manuscript (see page 13, the last paragraph, and page 22, the second paragraph).

The reason that telomerase could not contribute to more extensive telomere lengthening in the ogg1Δ rad52Δ clones is probably due to the fact that in some of those clones telomere lengthening is totally Rad52p-dependent as discussed in page 12, the last paragraph.

**Telomere position effect and cell growth of telomerase deficient strain**

We agree with the reviewer that the TPE effect in ogg1Δ strain is not significant and have stated this in the revised manuscript (see page 16, the last paragraph). Because of fluctuations among experiments, we presented the data from independent experiments in Figure S9.

We have repeatedly observed deletion of OGG1 delayed the loss of cell viability in some est2 mutant clones. According to the reviewer’s suggestion, we have replaced the original streak data with the spotting of serial dilutions of culture for better clarification of the difference among the mutants (Figure 7). Deletion of the EST2 gene leads to loss of cell viability at day 4, while deletion of the OGG1 gene can delay this phenotype in some est2 mutant clones. This observation suggests that deletion of OGG1 has triggered a rescue pathway. Deletion of OGG1 in est2Δ rad52Δ strains did not lead to prolonged cell viability. Taken together, these results indicate that Ogg1p may act in Rad52-pathway in regulating cellular senescence of est2 deletion mutant.
To address the reviewer’s question on Fig 7B of previous version, we repeated the ogg1Δ est2Δ experiment with more independent clones, and confirmed that some ogg1Δ est2Δ clones exhibited a heterogeneous telomere length population with some telomeres being longer and some shorter than that in est2Δ clone (Figure S5A) as discussed in page 12, the last paragraph.

**Rap1’s binding in ogg1 mutants**

We followed the reviewer’s comments and added the data showing that expression of Rap1 and Rif2 and their pull-down efficiency for Chip analysis were similar in wild type and ogg1Δ strains (Figure S8).

It is known that telomerase-mediated telomere elongation is negatively regulated by the number of Rap1p complex in telomeres, i.e. the more Rap1p in telomeres, the less telomere repeats added by telomerase. Our genetic analysis demonstrates that the telomerase pathway participates in moderate telomere lengthening in ogg1Δ strain, and the CHIP analysis shows that the association of Rap1p and Rif2p to telomeric DNA is moderately decreased in ogg1Δ strain. Our results therefore support that increased telomerase-mediated telomere elongation is likely the results of decreased number of telomere protein complex in telomeres.

We agree with the reviewer’s point that it may be rare that two adjacent Rap1p binding sites are oxidatively damaged in vivo. However, the in vitro experiment was intended to test if increased number of affected Rap1p-binding sites may account for greater loss of Rap1p’s affinity to telomeres. This finding is consist with the previous in vitro observation by Opresko et al that the amount of 8-oxoG in telomere repeat sequences affects the binding of mammalian telomere binding proteins, TRF1 and TRF2 to the telomeric DNA (Nucleic Acids Res, 33, 1230-1239, 2005). In fact, the presence of 8-oxoG in one of two Rap1p-binding sites is enough to cause approximately 3-fold decrease in the affinity of Rap1p to telomeres. This correlates with the mild reduction of in vivo Rap1 binding and the mild telomere lengthening phenotype observed in ogg1Δ strain. Oxidative base lesions may be more frequent in some telomeres than others. Southern blot analysis shows that telomere lengthening is unequal in ogg1Δ strain, with telomere size ranging from wild type to longer than wild type. PCR analysis also confirms that telomere lengthening is unequal or does not occur in single telomeres (chromosome 6 right arm and 15 left arm). These results suggest that not all the telomeres are affected by oxidative base damages. It is possible that a telomere that harbors more lesions may have less telomere-bound Rap1p and thus more access of telomerase to add more telomere repeats. Differential damage among telomeres could be the bases for uneven telomere lengthening.

**Minor comments:**

We have omitted the word of “individual” telomeres in the discussion. We referred chromosome 6 right arm and 15 left arm as single telomere throughout the manuscript.

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**2nd Editorial Decision** 28 October 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original three referees to review the revised manuscript and I have now heard back from them. As you can see below, all three referees find that their original raised concerns have been satisfactorily addressed (Referee #2 has no further comments to the authors). I am therefore pleased to proceed with the acceptance of the paper for publication here. Before doing so, referee #3 has a few minor comments concerning the interpretation of some of the data and I would like to ask you to respond to those 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.

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Referee #1 (Remarks to the Author):

I am satisfied with the changes that were made.

Referee #3 (Remarks to the Author):

The additional experiments add rigor to the conclusions and significantly improve the manuscript. However, while the authors convincingly show that loss of Ogg1 leads to telomere lengthening (as shown previously), and inhibit Rap1 and Rif2 binding at the telomeres, there is no direct evidence that this is due to oxidative damage at the telomeres in vivo. Never-the-less the authors are careful not to over state their results, and the novelty lies in the fact that Ogg1 deletion results in telomere lengthening through telomerase and/or recombination pathways, and suppression of Rap1 and Rif2 binding. The authors raise an important point in the discussion that the type of oxidative background may determine the impact on telomeres (i.e. lengthening versus shortening). In this light, it is also critical to note that the genetic background is important. The authors did not see telomere lengthening after treating wild type cells with hydrogen peroxide. This is important and should be included as "data not shown". Some reports in the literature indicate the intermediates in DNA repair may be more toxic than the lesion itself (Sobol et al JBC, 2003; Meira et al PNAS 2005). Therefore, the ssDNA breaks that result from Ogg1 lyase activity incising the backbone at sites of oxidized bases may impact the telomere differently (i.e. cause shortening (Richter et al, MAD 2007; Petersen et al Exp Cell Res, 1998)) than the oxidized bases themselves. In the absence of Ogg1 then there should be less incision of oxidized bases and thus, fewer ssDNA breaks. It is important to note this in the discussion.

The data that show deletion of Tsa1 causes telomere lengthening is interesting, but one cannot necessarily assume this is due to increased damage at the telomeres; unless it is directly shown to be the case. For example, Askree et al (PNAS) showed that a wide range of genes influence telomere length, presumably by a variety of different mechanisms. This caveat should be mentioned in the results or discussion.

2nd Revision - authors’ response 02 November 2009

Answers to Reviewer #3’ comments:

We thank the reviewer’s appreciation of our additional experiments and modifications to the manuscript. We addressed the comments by the reviewer as follows:

1. We included the statement that “non-lethal doses of H₂O₂ did not lead to telomere length change in wild type and ogg1Δ strain (data not shown)” (see page 8, lines 12-13).
2. We added the discussion of DNA repair intermediates in affecting telomere length “Single stand DNA breaks (SSBs) resulting from Ogg1 lyase activity (i.e. incising the backbone at sites of oxidized bases) may also impact telomere length differently than the oxidized bases themselves. In the absence of Ogg1, there may be less incision of oxidized bases and thus, fewer SSBs and less telomere attrition” (see page 20, lines 12-15).
3. We included the statement that “Although deletion of TSA1 may affect telomeres length via the mechanisms other than telomere DNA damage, these data nevertheless imply that increased endogenous oxidative stress can exacerbate telomere lengthening in ogg1Δ strain” (see page 8, lines 19-21).