Rap1 prevents fusions between long telomeres in fission yeast

Lili Pan, Duncan Tormey, Nadine Bobon and Peter Baumann
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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Thank you for submitting your manuscript on vulnerability of long telomeres for our consideration, and apologies for the delay in its evaluation due to the holiday season. I have now heard back from three expert referees, whose comments are copied below. As you will see, the referees find your results and the concept emerging from them potentially interesting. At the same time, all of them presently retain significant reservations regarding the decisiveness of the data supporting the key conclusions, and the depth of understanding.

Among the issues raised, I would consider the following particularly crucial:

- Comparison of long and short, not just long and normal, telomeres (ref 1 pt 1)
- Follow-up analysis into the role of telomeric ssDNA overhangs (ref 2 pt 1, ref 1 pt 8)
- Confirming the causal role of telomere length as opposed to shelterin disruption (ref 2 pt 2)
- Quantitative assessment of telomere binding and of Bqt4 interaction of Rap1 mutants (ref 3 main point)

Should you be able to satisfactorily address these key concerns, as well as the various other, more specific points noted by referees 1 and 2, we would be interested in pursuing a revised manuscript further for EMBO Journal publication. Please be reminded that it is our policy to allow only a single round of (major) revision, making it important to carefully respond to all points raised at this point. Also, please do not hesitate to contact me in case you should require an extension of the revision period, or if you would like to discuss/clarify plans for addressing the main issues ahead of resubmission.

Detailed information on preparing, formatting and uploading a revised manuscript can be found below and in our Guide to Authors.

Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to your revision!
Referee #1:

Comments to the authors

In this manuscript, Pan and co-workers propose a new concept of genome integrity. It has been thought that critically short telomeres are the biggest targets of mistaken DNA repair; however, long telomeres also induce chromosome end fusions. Mutagenesis and partial deletions of the rap1 gene in S. pombe reveal that tethering of long telomeres at the nuclear envelope is involved in the protection of chromosome ends. This study provides detailed insights into the relationship between telomere length and chromosome end fusion events. The following points should be addressed before publication.

Major points:

1) The word, "short (telomeres)" is misleading. In this manuscript, only the long and "normal" length of telomere DNA were analyzed.

2) In regard to the point above, telomeres shorter than the wild-type length (critically short telomeres) should be analyzed, if the authors claim that long telomeres are "more likely" to undergo end fusions compared with "critically short telomeres" (page 5, lines 97-100).

3) In this manuscript, the Rap1 protein level in each strain should be presented. Otherwise, the PFGE data cannot be interpreted precisely. For example, in page 6, line 131, there is a sentence "Western analysis ... on all constructs showed ..."; however, Figure S1 only shows Rap1 expressions in several strains. Similarly, Rap1 expressions in bqt4 or bqt3 deletion strains should be presented. If Rap1 expression is remarkably low in some strains, it will be inappropriate to conclude that some parts of Rap1 are important for end protection.

4) The control, Rap1-delta RCT, is missing from Figure 2 (panels C and D).

5) It is difficult to understand how telomere foci distribution was measured in Figure 7 (panel A). More explanations are required in Figure 7A and Materials and Methods. It seems that the authors did not select focal planes with brightest "Nup44-mCherry" signals. If so, distances between telomeres and the nuclear envelope cannot be directly measured with microscopic data on one focal plane (needs for calculations).

6) Figure 7 (panels C and D) also needs more explanations and careful discussion. In these panels, the samples were collected without re-streaks? Panel C shows absolutely high background; thus, deletions of Bqt4 or Bqt3 do not result in the similar levels of end fusions as those observed in the various rap1 mutants, suggesting that tethering of telomeres to the NE plays only a minor role in end protection of long telomeres. The main text in results and discussion needs revisions.

7) References are not appropriately cited. The authors should re-consider citations carefully. For example, Fujita et al. (PLoS ONE, 2012) have already shown that the poz1 deletion strain exhibits protected chromosome ends and the deletion of Rap1 456-513 results in unprotected chromosome ends and elongated telomeres, and that Taz1 interacts with the RCT domain of Rap1. Release of telomeres from the nuclear envelope in the bqt4 deletion strain has been shown in Fujita et al. (Curr. Biol., 2012).
8) In the rap1-delta strain, telomeres are mostly ssDNA (Miller, Nature, 2006). It may be good to discuss about involvement of the long ssDNA structure in the end protection dependent on telomere tethering to the NE.

Minor points:

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3) Figure legends needs more explanations, even if some descriptions are included in the main text. For example, how they collected samples (nitrogen starvation/G1 arrest, how many streaks, etc.) should be described in each panel because readers can easily misunderstand.

4) Description of figure numbers (Figure S1, etc.) is sometimes missing from the main text.

5) There are two versions of Rap1-tagging; N-terminal and C-terminal. Explanation for the reason is required. (It is better to present the comparison of Rap1 expression levels of both tagging.)

Referee #2:

Pan et al study the role of Rap1 in preventing telomere fusions in S. pombe. Using a set of deletion mutants, they map the domains required to the RCT and the region immediately N-terminal to this. A mutagenesis library refines this further to identify a number of novel mutations outside of the RCT. One set of these mutations cluster within the Bqt-binding motif (also called p-patch) and one is outside, F545L. Interestingly, the extent to which these rap1 mutants prevent telomere fusion is influenced by telomere length - chromosome fusions are less likely as telomeres become shorter over time. This effect can also be seen by deletion of poz1, which causes longer telomeres and increased fusions. The authors show that mutation of the p-patch (M6) but not F545L mislocalises telomeres away from the nuclear periphery in G1.

These observations are interesting and they point towards a novel means by which excessively long telomeres can be deleterious.

1. I feel that the main weakness of this paper is the lack of a mechanism to explain their observations. To my mind, the obvious candidate is an effect on telomeric overhangs. So, in a sense the length of telomeric dsDNA (as measured in the Southern blots) might be a red herring. What might be more relevant to preventing NHEJ-mediated fusions, is the length of the telomeric ssDNA overhangs. I feel the paper would be significantly strengthened if this were measured directly and an assessment made of how rap1 mutants interact genetically with pot1 with regards to fusions?

2. Are the increased chromosome fusions seen in rap1 mutants coincident with long telomeres or caused by long telomeres? Can you increase the baseline levels of fusions in a rap1Δ strain by lengthening its telomeres still further? The results of bqt3Δ +/- poz1Δ argue for a causal effect of telomere length. However, there is always the risk of the effect being due to disrupting shelterin rather than increasing telomere length. It's not possible to completely avoid this risk but evidence for a causal effect of telomere length would be significantly strengthened if the authors could show a similar effect on fusions when lengthening telomeres using non-shelterin mutants e.g pmt3Δ, pst2Δ or vip1Δ.

3. I feel that the conclusions regarding telomere localisation are over-interpreted based on the data presented. The text states that "Nuclear envelope attachment mediates the protection of long telomeres". The data in Fig7B with rap1 F545L shows that this statement is not true or rather is too broad. Moreover, it is unclear to me whether the microscopy in Fig7 was performed in early passage rap1-M6 mutants (with long telomeres and high levels of chromosome fusions) or in late passage mutants (with short telomeres and low levels of chromosome fusions). Ideally, chromosome fusions and telomere length would be quantified in the same cells as being imaged by microscopy. Without this, one cannot assess potential correlations.

Referee #3:

In the current manuscript, Pan and coauthors set up to study the role exerted by fission yeast Rap1 in end protection. While it is well established that yeast cells lacking Rap1 accumulate telomeric fusions when they are arrested in G1, the mechanistic details of how Rap1 suppresses fusions are not well established. By analyzing deletion and point mutants of Rap1, the authors discovered that long telomeres are more susceptible to NHEJ-mediated fusions and that long telomeres are likely to be
protected by fusions by being sequestered to the nuclear envelope through Rap1-buquet interactions. Indeed, deletion of Bqt3 or Bqt4 phenocopy the expression of the identified Rap1 mutants.

Overall, this paper is of very high quality. The data here presented are solid and the interpretation is careful enough. The concept of long telomeres requiring different mechanisms of protection than short ones is intriguing and for sure will open new avenues of research. I'm therefore in favor of considering this work for publication.

However, I found that a major pitfall is associated with the data and this has to do with a lack of analysis of how the different Rap1 mutants bind to telomeres. This is such an essential point. The authors should perform chip experiments to carefully quantify the density of the different Rap1 mutants at telomeres (short and long, possibly also in a taz1-delta background as one cannot exclude Taz1 independent binding of Rap1 to telomeres). As said, this is essential; in the end the authors need to make sure that what they observe is not simply the result of some unpredicted effects on protein conformation or similar.

It would also be essential, in my opinion, to verify biochemically that the p-patch mutants do not interact properly with Bqt4, as this also seems a central assumption of the proposed model.
Re: EMBOJ-2021-110458

Specific vulnerability of long telomeres to undergo end fusions revealed by mutational analysis of Rap1

Authors response to Referee’s comments shown in green

Referee #1:

Comments to the authors

In this manuscript, Pan and co-workers propose a new concept of genome integrity. It has been thought that critically short telomeres are the biggest targets of mistaken DNA repair; however, long telomeres also induce chromosome end fusions. Mutagenesis and partial deletions of the rap1 gene in S. pombe reveal that tethering of long telomeres at the nuclear envelope is involved in the protection of chromosome ends. This study provides detailed insights into the relationship between telomere length and chromosome end fusion events. The following points should be addressed before publication.

We thank reviewer 1 for highlighting that the vulnerability of long telomeres is “a new concept of genome integrity” and for referring to our study as providing “detailed insights into the relationship between telomere length and chromosome end fusion events.” The points raised by the reviewer to further improve the manuscript have prompted us to carry out additional experiments now included in this revised version.

Major points:

1) The word, "short (telomeres)" is misleading. In this manuscript, only the long and "normal" length of telomere DNA were analyzed. It was not our original intention to compare the vulnerability of short and long telomeres, but rather to point out that long telomeres can also be vulnerable to undergoing fusions. We have modified the text accordingly to make it clear that we are comparing long telomeres with those within the normal length distribution. However, the reviewers’ comment encouraged us to indeed examine short telomeres in the context of the Rap1 mutant as well. See below.

2) In regard to the point above, telomeres shorter than the wild-type length (critically short telomeres) should be analyzed, if the authors claim that long telomeres are "more likely" to undergo end fusions compared with "critically short telomeres" (page 5, lines 97-100).

While a direct comparison of long and short telomeres was not our intention, we realize that the statement referred to by the reviewer gave this impression. We therefore analyzed the Rap1 mutants in a ‘critically short telomere’ background mediated by deletion of pof8. We now show in the new Figure EV4 that end protection is indeed partially compromised in Rap1 p-patch and F545L mutants in the absence of pof8, but the level of end-fusions is not as high as when telomeres are long. Although this data supports the earlier statement, we do not see a comparison of vulnerabilities of short versus long telomeres as central to this manuscript. The key point we would like to make is that long telomeres are vulnerable, unlike the common assumption that telomere erosion is the path to uncapping. Instead, we propose that end fusion and genome instability lurk on both sides of an ‘optimal’ telomere length.
3) In this manuscript, the Rap1 protein level in each strain should be presented. Otherwise, the PFGE data cannot be interpreted precisely. For example, in page 6, line 131, there is a sentence "Western analysis ... on all constructs showed ..."; however, Figure S1 only shows Rap1 expressions in several strains. Similarly, Rap1 expressions in bqt4 or bqt3 deletion strains should be presented. If Rap1 expression is remarkably low in some strains, it will be inappropriate to conclude that some parts of Rap1 are important for end protection.

The “on all constructs” in the sentence on page 6, line 131, refers to the presence of the C-terminal V5-epitope tag. This is important as we and others have found that comparisons between tagged and untagged proteins can be problematic due to (sometimes small) effects of tags on protein function that are compounded by mutations elsewhere in the protein (e.g. for Pot1 and Trt1). But this minor point aside, we fully agree with the reviewer that if a part of the protein is mutated or deleted, and this version of the protein is expressed at low levels, it would be inappropriate to conclude that the mutation is directly affecting end protection, rather than the alternative explanation of the lower protein level being responsible for the defect. In this case, an expression level analysis is essential. On the other hand, if part of a protein is deleted, for example, Rap1_249-693 in Figure 1C, without causing an end-fusion phenotype, it seems reasonable to conclude that the region is not important, especially when a further truncation 440-693 is still able to provide protection even though the steady-state level of 440-693 is lower than wildtype (Figure 1D and Appendix Figure S1).

Since we observed end-fusion phenotype with the Rap1 p-patch mutant and F545L mutant in poz1Δ background (Figure 6C) and in ppm1Δ and pof8Δ background (new Figure 6D and Figure EV4), we added expression analysis of those strains in comparison to wildtype Rap1. In all cases, the mutants were expressed at higher level than the wildtype protein. We also analyzed Rap1 protein levels in bqt4 or bqt3 deletion alone, and in addition to poz1 deletion background. The western blot data is in new Appendix Figure S5C-F.

4) The control, Rap1-delta RCT, is missing from Figure 2 (panels C and D).

We have previously published that telomeres are elongated in Rap1-ΔRCT similar to rap1Δ (Pan, Genes and Dev. 2015). Figure 2C shows that a Rap1ΔRCT-Taz1 fusion rescues telomere length to WT levels, confirming the recruitment role for the RCT domain. The Rap1ΔRCT-Poz1 fusion is used as a negative control because Rap1 recruitment to telomeres is dependent on the Rap1-Taz1 interaction via the RCT domain, whereas the Rap1-Poz1 interaction is insufficient to recruit functional levels of Rap1 to telomeres.

In Figure 1B, we show that Rap1ΔRCT strains suffers end fusions and this phenotype is rescued when Rap1ΔRCT is fused to Taz1, confirming that the RCT domain functions in recruitment, but is not itself required to prevent end fusions.

5) It is difficult to understand how telomere foci distribution was measured in Figure 7 (panel A). More explanations are required in Figure 7A and Materials and Methods. It seems that the authors did not select focal planes with brightest "Nup44-mCherry" signals. If so, distances between telomeres and the nuclear envelope cannot be directly measured with microscopic data on one focal plane (needs for calculations).
We selected focal planes with the brightest telomere foci (Pot1-GFP or Taz1-GFP) but excluded the top and bottom focal planes where no crisp Nup44-mCherry ring signal is observed. Even though this does not always select the most central plane, the ratio of the distance between telomere focus and NE to the distance between the center and the NE gives a representation of distance from the periphery. This method was described by the Gasser group in budding yeast (Hediger et al., 2002) and has also been used in S. pombe (Maestroni et al., 2020). We have modified Figure 8A (new number) and added additional details explaining the methodology in the figure legend.

6) Figure 7 (panels C and D) also needs more explanations and careful discussion. In these panels, the samples were collected without re-streaks? Panel C shows absolutely high background; thus, deletions of Bqt4 or Bqt3 do not result in the similar levels of end fusions as those observed in the various rap1 mutants, suggesting that tethering of telomeres to the NE plays only a minor role in end protection of long telomeres. The main text in results and discussion needs revisions.

In figure 7C, the strains were generated by knocking out bqt4 or bqt3 in WT or poz1Δ strain, the transformation colonies were then restreaked 3 times. For Figure 7D, the cells were collected after two additional restreaks. Because deletion of bqt3 or bqt4 in poz1Δ background doesn’t further affect telomere length, collecting cells at different time points was not necessary as telomere length is at steady-state. The levels of end-fusions in bqt4Δ or bqt3Δ are indeed lower than in the Rap1 mutants. This supports the conclusion that more than one mechanism is involved as originally stated in the last paragraph of the result section. We have revised the text to make this clearer.

7) References are not appropriately cited. The authors should re-consider citations carefully. For example, Fujita et al. (PLoS ONE, 2012) have already shown that the poz1 deletion strain exhibits protected chromosome ends and the deletion of Rap1 456-513 results in unprotected chromosome ends and elongated telomeres, and that Taz1 interacts with the RCT domain of Rap1. Release of telomeres from the nuclear envelope in the bqt4 deletion strain has been shown in Fujita et al. (Curr. Biol., 2012).

We have reviewed the citations and updated them accordingly. Whenever possible, we now reference multiple papers when related findings were published by different labs.

8) In the rap1-delta strain, telomeres are mostly ssDNA (Miller, Nature, 2006). It may be good to discuss about involvement of the long ssDNA structure in the end protection dependent on telomere tethering to the NE.

We have now analyzed the single-stranded telomere lengths using duplex-specific nuclease (DSN) as described in Zhao et al, (2011) Methods Mol Biol. Our analysis shows similar single-strand signals for Rap1 mutants and WT Rap1 (New Figure 7C). This argues against a model in which the Rap1 mutations result in the loss of the single-stranded overhang. It has been shown in mammalian cells that single stranded overhangs must be removed before NHEJ-mediated end-fusions when telomeres are dysfunctional due to dominant negative TRF2 or senescence (Smogorzewska, 2002 Curr Biol, Stewart 2003 Nature Genetics). Therefore, an elongated single stranded overhang in long telomere mutants would be expected to inhibit end-fusions rather than promote them.
Minor points:

1) There are some mistakes in fonts or spacing. For example, page 6, line 118, delta of rap1-delta and line 124, "delta (lanes 6 and 7)" should not be in italic. Page 8, lane 178; S. pombe (space), etc.

We have corrected these mistakes.

2) In Figure 1, it will be helpful for readers' understanding if there is a schematic illustration of Not1 cutting sites at the ends of chromosomes 1 and 2 (and no cutting site in chromosome 3).

This illustration has been added as new Figure 1B.

3) Figure legends needs more explanations, even if some descriptions are included in the main text. For example, how they collected samples (nitrogen starvation/G1 arrest, how many streaks, etc.) should be described in each panel because readers can easily misunderstand.

We have added additional descriptions in the figure legends.

4) Description of figure numbers (Figure S1, etc.) is sometimes missing from the main text.

We have verified that all figure panels are described in the main text.

5) There are two versions of Rap1-tagging; N-terminal and C-terminal. Explanation for the reason is required. (It is better to present the comparison of Rap1 expression levels of both tagging.)

We tested that both N- and C-terminally tagged Rap1 show WT telomere length and end protection. While strains in Figure 1C were C-terminally tagged, we used N-terminally tagged Rap1 for later strain construction. Western blot analysis showed a stronger signal for C-terminally tagged Rap1 than for N-terminally tagged Rap1, this may reflect tag accessibility or differences in expression level. Importantly, in all experiments we only compare mutants to WT carrying the same tag.

Referee #2:

Pan et al study the role of Rap1 in preventing telomere fusions in S. pombe. Using a set of deletion mutants, they map the domains required to the RCT and the region immediately N-terminal to this. A mutagenesis library refines this further to identify a number of novel mutations outside of the RCT. One set of these mutations cluster within the Bqt-binding motif (also called p-patch) and one is outside, F545L. Interestingly, the extent to which these rap1 mutants prevent telomere fusion is influenced by telomere length - chromosome fusions are less likely as telomeres become shorter over time. This effect can also be seen by deletion of poz1, which causes longer telomeres and increased fusions. The authors show that mutation of the p-patch (M6) but not F545L mislocalises telomeres away from the nuclear periphery in G1.

These observations are interesting and they point towards a novel means by which excessively long telomeres can be deleterious.
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We thank referee 2 for his interest in our observation that telomere length influences the likelihood of fusions occurring and stating that this points “towards a novel means by which excessively long telomeres can be deleterious.” Prompted by the referees suggestions for further mechanistic studies, we have now examined overhang length using duplex-specific nuclease (DSN) (Zhao et al, 2011 Methods Mol Biol). Our analysis showed similar signals for single-stranded G-strand overhangs between Rap1 WT protein and the mutants and no C-strand signals in any of the strains (New Figure 7C). While this does not nail the mechanism underlying the vulnerability of long telomeres it argues against the hypothesis noted by the reviewer. Considering these results, we did not pursue possible genetic interactions with Pot1, since Rap1 mutants cause end-fusions via NHEJ, whereas pot1Δ results in rapid loss of telomeres and chromosome circularization mediated by the single-strand annealing pathway (Wang et al, Mol. Cell 2008). We agree that understanding the underlying mechanism is now important, but in light of the amount of data presented in this revised version of the manuscript, we feel that further studies towards a molecular mechanism are beyond the scope of this paper.

2. Are the increased chromosome fusions seen in rap1 mutants coincident with long telomeres or caused by long telomeres? Can you increase the baseline levels of fusions in a rap1Δ strain by lengthening its telomeres still further? The results of bqt3Δ +/- poz1Δ argue for a causal effect of telomere length. However, there is always the risk of the effect being due to disrupting shelterin rather than increasing telomere length. It's not possible to completely avoid this risk but evidence for a causal effect of telomere length would be significantly strengthened if the authors could show a similar effect on fusions when lengthening telomeres using non-shelterin mutants e.g pm3Δ, pst2Δ or vip1Δ.

We agree that this is an important distinction even though indirect effect on shelterin cannot be ruled out. We appreciate the suggestion. We have now added data for ppm1Δ, a non-shelterin protein with very long telomeres similar to poz1Δ. We did not use pm3Δ or pst2Δ as they have been shown to affect protein interaction or transcript length of a shelterin component (Garg et al 2014, Miyagawa et al 2014, Choi et al 2012) and have milder telomere elongation phenotypes (Liu et al 2010). When cells have long telomeres due to the deletion of ppm1, a leucine carboxyl methyltransferase that is not part of the shelterin complex, the long telomeres show the same sensitivity towards uncapping and fusion as observed for poz1Δ. This data is shown in new figure 6D.

3. I feel that the conclusions regarding telomere localisation are over-interpreted based on the data presented. The text states that "Nuclear envelope attachment mediates the protection of long telomeres". The data in Fig7B with rap1 F545L shows that this statement is not true or rather is too broad. Moreover, it is unclear to me whether the microscopy in Fig7 was performed in early passage rap1-M6 mutants (with long telomeres and high levels of chromosome fusions) or in late passage mutants (with short telomeres and low levels of chromosome fusions). Ideally, chromosome fusions and telomere length would be quantified in the same cells as being imaged by microscopy. Without this, one cannot assess potential correlations.
The misleading phrase in the text has been changed. It was never our intention to portray nuclear envelope attachment as THE mechanism that mediates protection of long telomeres. Indeed, we already noted that F545L must mediate fusions of long telomeres independent of NE attachment. This is now more clearly stated.

The rap1_ M6 and F545L mutants used in Figure 7 were generated by directly replacing endogenous rap1 with rap1 mutants so the telomere length was not changed in the process – they stay very long. The purpose of the microscopy was to check whether the M6 and F545L mutations affect NE association via Bqt4, providing a possible mechanism that leads to deprotection. We have now further confirmed the effects of the mutations on the interaction between Rap1 and Bqt4 by yeast two hybrid assay. This data is now included as new figure 8C and strengthens the previous conclusion.

Referee #3:

In the current manuscript, Pan and coauthors set up to study the role exerted by fission yeast Rap1 in end protection. While it is well established that yeast cells lacking Rap1 accumulate telomeric fusions when they are arrested in G1, the mechanistic details of how Rap1 suppresses fusions are not well established. By analyzing deletion and point mutants of Rap1, the authors discovered that long telomeres are more susceptible to NHEJ-mediated fusions and that long telomeres are likely to be protected by fusions by being sequestered to the nuclear envelope through Rap1-buquet interactions. Indeed, deletion of Bqt3 or Bqt4 phenocopy the expression of the identified Rap1 mutants. Overall, this paper is of very high quality. The data here presented are solid and the interpretation is careful enough. The concept of long telomeres requiring different mechanisms of protection than short ones is intriguing and for sure will open new avenues of research. I'm therefore in favor of considering this work for publication.

However, I found that a major pitfall is associated with the data and this has to do with a lack of analysis of how the different Rap1 mutants bind to telomeres. This is such an essential point. The authors should perform chip experiments to carefully quantify the density of the different Rap1 mutants at telomeres (short and long, possibly also in a taz1-delta background as one cannot exclude Taz1 independent binding of Rap1 to telomeres). As said, this is essential; in the end the authors need to make sure that what they observe is not simply the result of some unpredicted effects on protein conformation or similar.

It would also be essential, in my opinion, to verify biochemically that the p-patch mutants do not interact properly with Bqt4, as this also seems a central assumption of the proposed model.

We thank reviewer 3 for praising the quality of the data and stating that our key observation will “for sure open new avenues of research”. We appreciate the concern that the Rap1 mutations may result in differences in occupancy at telomeres. As suggested, we have performed ChIP experiments to compare telomere binding of Rap1 mutants to the wildtype protein in wildtype as well as in long telomere backgrounds caused by deletion of poz1 or ppm1, respectively. The amount of telomeric DNA recovered with wt or mutant Rap1 was similar. This data is now shown as new Figure 7A and 7B. While we cannot rule out that small differences in Rap1 occupancy affect protection, this does not appear to be the main mechanism that explains vulnerability of long telomeres.

We have also performed yeast two hybrid to directly assess the interaction between Bqt4 and the p-patch mutant (M6) as well as the F545L mutant. Consistent with the microscopy, the M6 mutant fails to
interact with Bqt4, whereas F545L retains the ability to bind. Both mutants bind to Taz1, further supporting that the loss of interaction between Rap1_M6 and Bqt4 is not due to misfolding of the M6 mutant protein. This data is now shown as new Figure 8C.
Thank you for submitting your revised manuscript to The EMBO Journal. We have now heard back from the three original referees, who have assessed the study once more and were, I am happy to say, fully satisfied with the revisions. As soon as a few remaining editorial points listed below have been addressed, we shall therefore be ready to accept the study for publication.

- Please adjust the format of the Data Availability section (including direct links to the deposited datasets, and removal of reviewer information) along the lines stipulated in our Guide to Authors: https://www.embopress.org/page/journal/14602075/authorguide#dataavailability

- I would suggest to rephrase the title to make it somewhat more explicit and appealing for a wider audience, maybe by turning it into the active voice, e.g. along the lines of "Mutational analysis of (fission yeast) Rap1 reveals that long telomeres are particularly/uniquely prone/vulnerable to end fusions" [although I am not even sure whether it is crucial to mention Rap1 mutational analysis in the title?]

- Finally, please upload suggestions for a short 'blurb' text prefacing and summing up the study in two sentences (max. 250 characters), followed by 3-5 one-sentence 'bullet points' with brief factual statements of key results of the paper; they will form the basis of an editor-written 'Synopsis' accompanying the online version of the article. Please see the latest research articles on our website (embojournal.org) for examples about the format of these synopses. Please also upload a synopsis image, which can be used as a "visual title" for the synopsis section of your paper. The image should be in PNG or JPG format with the modest dimensions of 550 pixel wide x 300-600 pixel high.

I am therefore returning the manuscript to you for a second round of revision, solely to allow you to incorporate the requested editorial modifications, and upload all modified files. Once we will have received the re-revised files, we should be ready to swiftly proceed with acceptance and publication of the manuscript.

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Referee #1:

The manuscript has been sufficiently improved according to the reviewers' comments.
Referee #2:

As stated in the rebuttal letter, the authors argue that "long telomeres are vulnerable, unlike the common assumption that telomere erosion is the path to uncapping. Instead, we propose that end fusion and genome instability lurk on both sides of an 'optimal' telomere length."
The data presented in their manuscript is consistent with this statement and I am happy that the authors have further strengthened their supporting evidence with a number of key new figures.

Referee #3:

I appreciate the work done by the authors to address the concerns I raised. I find this manuscript suitable for publication in The EMBO Journal.
Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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**Corresponding Author Name:** Peter Baumann
**Journal Submitted to:** EMBO Journal

### Materials and Methods

> Materials and Methods, Appendix Table S2, S3

> Not Applicable

> Information included in the

> Materials and Methods, Appendix Table S5

> - definition of error bars as s.d. or s.e.m.
> - definition of ‘center values’ as median or average;
> - exact statistical test results, e.g., P values = x but not P values < x;
> - are there adjustments for multiple comparisons?
> - are the assay(s) and method(s) used to carry out the reported observations and measurements.
> - the exact sample size (n) for each experimental group/condition, given as a number, not a range;
> - a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.);
> - a statement of how many times the experiment was independently replicated in the laboratory.

**Source Data** Should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

### Abridged guidelines for figures

#### 1. Data

- The data shown in figures should satisfy the following conditions:
- The data were obtained and processed according to the field’s best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- Clearly, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- OR, if preferred, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Please note that a copy of this checklist will be published alongside your article.

#### 2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (e.g., cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/variable/modified in a controlled manner.
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment was independently replicated in the laboratory.

**Newly Created Materials**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Yes                                    | Materials and Methods, Appendix Table S2, S3, S4, S5 |

**Antibodies**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Yes                                    | Materials and Methods                          |

**DNA and DNA sequences**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Yes                                    | Materials and Methods, Appendix Table S5      |

**Cell lines**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable                         | (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |

**Plants and microbes**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Not Applicable                         | (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |

**Human research participants**

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|----------------------------------------|-----------------------------------------------|
| Not Applicable                         | (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |

**Core facilities**

| Information included in the manuscript? | In which section is the information available? |
|----------------------------------------|-----------------------------------------------|
| Yes                                    | Acknowledgments                               |
| Study protocol | Information included in the manuscript? | In which section is the information available? |
|----------------|----------------------------------------|---------------------------------------------|
| If study protocol has been pre-registered, provide DOI in the manuscript. For clinical trials, provide the trial registration number OR cite DOI. | Not Applicable | (Protocol and Tools, Table, Materials and Methods, Figures, Data Availability Section) |
| Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), when applicable. | Not Applicable | (Protocol and Tools, Table, Materials and Methods, Figures, Data Availability Section) |

| Laboratory protocol | Information included in the manuscript? | In which section is the information available? |
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| Provide DOI OR other citation details if external detailed step-by-step protocols are available. | Not Applicable | (Protocol and Tools, Table, Materials and Methods, Figures, Data Availability Section) |

| Experimental study design and statistics | Information included in the manuscript? | In which section is the information available? |
|-----------------------------------------|----------------------------------------|---------------------------------------------|
| Include a statement about sample size estimate even if no statistical methods were used. | Yes | Materials and Methods, Figure legends |
| Have any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g., randomization procedure)? If yes, have they been described? | Not Applicable | |
| Include a statement about blinding even if no blinding was done. | Yes | Materials and Methods |
| Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | Yes | Materials and Methods, Figure legends |
| If sample or data points were censored from analysis, report if this was due to addition or intentional exclusion and justify. | |
| For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared? | Yes | Materials and Methods, Figure legends |

| Sample definition and in-laboratory replication | Information included in the manuscript? | In which section is the information available? |
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| In the figure legends, state number of times the experiment was replicated in laboratory. | Yes | Figure legends |
| In the figure legends, define whether data describes technical or biological replicates. | Yes | Figure legends |

| Ethics | Information included in the manuscript? | In which section is the information available? |
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| Studies involving human participants: State details of authority granting ethics approval (IRB or equivalent committee), provide reference number for approval. | Not Applicable | (Protocol and Tools, Table, Materials and Methods, Figures, Data Availability Section) |
| Studies involving human participants: Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | Not Applicable | |
| Studies involving human participants: For publication of patient photos, include a statement confirming that consent to publish was obtained. | Not Applicable | |
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| Dual Use Research of Concern (DURC) | Information included in the manuscript? | In which section is the information available? |
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| If you used a select agent, is the security level of the lab appropriate and reported in the manuscript? | Not Applicable | |
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| Reporting | Information included in the manuscript? | In which section is the information available? |
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| The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR. | Not Applicable | (Protocol and Tools, Table, Materials and Methods, Figures, Data Availability Section) |

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| For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link at top right). See author guidelines, under Reporting Guidelines. Please confirm you have followed these guidelines. | Not Applicable | |
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| Data Availability | Information included in the manuscript? | In which section is the information available? |
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| Have primary datasets been deposited according to the journal’s guidelines (see “Data Availability” section) and the respective accession numbers provided in the Data Availability Section? | Yes | Data Availability |
| Have human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | Not Applicable | Data Availability |
| Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Yes | Data Availability |
| If publicly available data were reused, provide the respective data citations in the reference list | Not Applicable | |