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Cancer-associated POT1 mutations lead to telomere elongation without inducing a DNA damage response

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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.)

1st Editorial Decision 18th Jan 2021

Thank you for submitting your manuscript on the effects of cancer-associated POT1 mutations in human cells to The EMBO Journal. I apologize for the delay in its evaluation, linked to both the holidays and to one referee having to be replaced due to illness. We have now finally received a full set of reports from three experts, and also had the chance to further discuss these slightly divergent reports within our team and with the reviewers. In light of these consideration, I am happy to invite you to revise this work based on the included reports, and to resubmit it using the link below.

As you will see from the comments copied below, referees 1 and 4 appreciate the importance of recreating and studying caPOT1 variants via genome engineering in a heterozygous context, while raising several well-taken specific concerns that I would ask you to address during revision. Referee 3 considers the conceptual advance less substantive based on previous studies such as Shi et al 2014 -something I would invite you to diligently discuss- and would thus expect follow-up insights into the molecular basis of caPOT1-mediated overlengthening, as well as into the causal role of increased telomere length for tumorigenesis in the caPOT1. Although any data you may be able to include to this effect would clearly be helpful, I realize that extensive experiments in this direction may go beyond the scope of a revision, and that adding thoughts on these points to the discussion may be sufficient. On the other hand, it will be important to directly address the more specific issues of referee 3, in particular the relevance of hESCs compared to HSCs (points 1 and 2) and the concern linked to the STELA assays (pt. 3) also mirrored in referee 4's comments. As it is our policy to allow only a single round of (major) revision, please do not hesitate to contact me in case you should have any question regarding the referee reports and how to best address them.

REFEREE REPORTS

Referee #1:

Shelterin is a six-subunit protein complex that functions at telomeric DNA to ensure telomere end protection. It is well established that defects in telomere end protection induce both telomere dysfunction and changes in telomere length, phenotypes often associated with cancer progression. Next generation sequencing has identified mutations in the shelterin protein POT1 in several cancers including, chronic lymphocytic leukemia, familial Hodgkin Lymphoma, and familial melanoma. The mutations in POT1 are often heterozygous missense or nonsense mutations that are not accompanied by loss of heterozygosity. However, exactly how these mutations contribute to the development of cancer in these patients is unclear. Here, the authors aim to dissect the cancer-associated POT1 (caPOT1) mutations using a combination of human embryonic, and hematopoietic, stem cells to determine whether telomere dysfunction or telomere elongation (or both) contribute to tumorigenesis. Using a CRISPR genome editing strategy the authors have recreated a number of cancer-associated mutations in POT1 and demonstrated that telomere elongation, not telomere dysfunction, is likely the cellular phenotype that promotes tumorigenesis. Overall, the data are convincing and the story makes an important contribution to our understanding not only of telomere biology, but also how defects in telomere maintenance contribute to the development of cancer. There are a few comments/concerns provided below.

Major Comments

1. The authors have attempted to recreate two known nonsense mutations in POT1 at position 214 and 250. The mutations that were generated are actually in/dels within exon 9. The authors argue that these in/dels resemble reported cancer-associated nonsense mutations at either amino acid 214 or 250. However, from the supplemental data the mutations appear to be around amino acid 226. Some numerical references would be helpful here to more precisely define the location of the in/dels. Combined, these in/dels have an allele frequency of about 25%, given that the 214 and 250 mutations are nonsense mutations, do these in/dels lead to any observable changes at the protein level? A WB for POT1 would be useful here.

2. At what point was telomere deprotection analyzed in the Q623H/WT and Q623H/ Q623H mutant cell lines? Does telomere dysfunction accumulate as telomere length increases?

3. How did the caPOT1 mutations affect telomere length in the HSC population?

4. The authors should include representative images of TIF in Figure 1b.

5. For ease of comparison can Figure 2a and Figure 2d be graphed the same?

6. In the text the authors state that they found two sgRNA that resulted in 20-25% gene editing. EV3 appears to demonstrate P1 and P2 were most efficient with nice data, however, the Figure legend for Figure 2a states that sgRNA-5 was used. sgRNA-1 is the label on the axis in the graph itself. I assume reference to sgRNA-5 was a typo?

7. The authors state that they confirmed the results of the long-term xenograft experiments of POT1-edited cells using the Q214X caPOT1 nonsense mutation generated with sgRNA-2. The EV aren't entirely clear, are the in/dels actually leading to a specific nonsense mutation at 214? Or is this a generalization for that region? The authors should clarify this point.

8. Figure 1 and 5 should be combined or discussed sequentially. The flow seems a bit odd with them separated by other data given that they are addressing the same question?

9. The authors conclude that telomere elongation drives the selection of caPOT1 mutations during cancer progression. However, this is overstated as the authors don't actually demonstrate cancer progression. The caPOT1 cells are not selected against and continue to contribute to the cellular population, but they aren't enriched over time and the authors have not analyzed tumorigenesis. The authors could refine the language here.

10. Figure 4a is already published data and while useful for the reader to understand the data presented in Figure 4b, seems like 4a and Table 1 should be combined and put in the supplement? Or a note included under Figure 4a noting that this is previously published data (similar to Table 1). The authors do this in the text, but within the Figure itself is also warranted.

Referee #3:

The role of POT1 mutations in various cancers is intriguing and worth exploring, and the question of whether POT1 mutations promote genome instability and thus facilitate tumorigenesis is not completely settled in the field. This study by Kim and co-authors presented their efforts in characterization of some cancer-associated POT1 (caPOT1) mutations in both human embryonic stem cells (hESCs) and hematopoietic stem cells (hCD34+) in a disease-relevant heterozygous setting. The authors claimed these caPOT1 mutants do not lead to telomere dysfunction-induced DDR, yet result in telomere lengthening, supporting the role of telomere lengthening in tumorigenesis. However, some caPOT1 mutants with these telomere phenotypes including A224A and S270A have been reported previously. In addition, other caPOT1 mutation, such as A532P, did not significantly lead to telomere lengthening. Thus, telomere lengthening may not be the sole basis of how POT1 mutations contributes to tumorigenesis. Nevertheless, it would be of great interests, if the authors explore (a) whether telomere lengthening is indeed in favor of tumorigenesis or not, for example, comparing the tumorigenesis potential of caPOT1 mutant cells with relatively short and long telomeres; and (b) how telomere lengthening contributes to tumorigenesis, for example, do caPOT1 mutant cells show any sign of replicative stress and genome instability? Without these additional experiments, their conclusion does not add novel mechanistic advancement to the topic. Some data interpretation and presentation also need further clarification (as follows).

1. That authors used hematopoietic stem cells (hCD34+) to test the effects of caPOT1 mutations is well accepted, given the close association of these mutations with hematopoietic tumors, such as CLL and lymphoma. However, the authors didn't justify the reason to use human embryonic stem cells (hESCs) as a cellular model. In fact, human stem cells respond to DNA damage very differently compared to somatic cells and lack certain cell cycle checkpoints (G1 and intra-S) ("Diminished apoptotic priming and ATM signaling confer a survival advantage onto aged hematopoietic stem cells in response to DNA damage". Nat Cell Biol. 2018 Apr; 20(4): 413-421"; "Attenuated DNA damage responses and increased apoptosis characterize human hematopoietic stem cells exposed to irradiation. Sci Rep 2018; 8, 6071"). In addition, a recent publication showed that uncapped hESCs exhibited a mutated damage response and can proliferate as wild type cells ("TRF2-mediated telomere protection is dispensable in pluripotent stem cells". Nature, Nov 25, 2020). Moreover, somatic cells with undetectable telomerase and limited telomere repeat reserve could hit the replicative senescence checkpoint, which acts as a tumor suppressor. Thus, telomere lengthening may help somatic cells overcome the replicative senescence checkpoint. In contrast, human stem cells express telomerase and have relatively longer telomeres. It is not known if hESCs

would hit the replicative senescence checkpoint in vivo. Thus, using stem cells to investigate POT1 mutations in regard to telomere-induced DDR and lengthening is not desirable. Somatic cell types may be considered, such as human melanocytes.

2. One of the major conclusions of the manuscript is that caPOT1 mutants do not lead to telomere dysfunction-induced DDR, measured by TIFs. However, the authors only test the TIF formation in hESCs, but not in any other cells including hematopoietic stem cells (with mutations at positions 214 and 250? Not clear to me), or Cas9-edited POT1 cells after bone marrow isolation as in Fig 3, as well as patient peripheral lymphocytes with Y36H mutation as in Fig 4.

3. The authors concluded that caPOT1 mutations lead to telomere elongation. However, throughout the manuscript only STELA is used to assess telomere length. STELA is a good method for single telomere length analysis, especially to detect a subpopulation of short telomeres, but lacking an overall measurement of bulk population of telomere length. To firmly conclude on telomere elongation, Southern blot of telomere restriction fragment analysis (TRF analysis) is required to provide more information on telomere length (e. g. length heterogeneity, overhang change, elongation rate, etc.).

4. In Fig 1, the authors stated in the texts that "... Y223C mutation showed a robust TIF response" and "All cells grow normally". First, growth curve of these cells compared to wild type cells should be included. Second, from Fig 1b, 2 POT1 Y223C/- hESCs showed 10-20% TIFs with high variation. Is this damage biologically meaningful? Does the "robust TIF response" cause any damage signaling and checkpoint activation?

5. In Fig 3, the authors evaluated the long-term proliferative capacity of caPOT1 mutations in engrafted mouse bone marrow cells. After transplantation, edited cells should be tested for TIF formation, chromosome aberrations by metaphase, and telomere length by TRF analysis or qFISH at different time points to address whether there is progressive telomere elongation and genome instability, compare to unedited cells. Also, comparing the editing frequency of POT1 in hCD34+ cells before and after engraftment, it increased from 12% to 23%. Is this change significant, representing a positive selection advantage for cell proliferation? Finally, did the immune deficient mice after engraftment show any hematopoietic lineage change in differentiation pattern, and more importantly, any occurrence of hematopoietic neoplasia?

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Minor points:

1. The Cas9-edited cells are clonal population. It is unclear in the manuscript where wt/wt cells used in STELA are pooled cells or clonal cells.

2. The authors introduced caPOT1 mutations into hematopoietic stem cells (hCD34+) at positions 214 and 250 without citing any reference or brief introduction of whether these two mutations have ever been characterized.

3. In Fig 5b, are wt/Y223C + TPP1 delPBD and wt/puro + TPP1 delPBD mislabeled?

Referee #4:

The manuscript "Cancer-associated POT1 mutations lead to telomere elongation without induction of a DNA damage response" examines cancer-associated POT1 mutations in a physiologically relevant context to address the important question whether induction of a DDR and associated genome instability or telomere elongation underlies the cancer-promoting property of these mutations. Prior work has provided support for both phenotypes based on overexpression studies. Given the exquisite importance of stoichiometries and absolute abundance of factors involved in telomere maintenance, a study that examines the effects of mutations in the context of genome-engineered heterozygosity was much needed and anticipated by the field. Using a combination of cell-based and long-term xenograft experiments, the authors demonstrate that none of the mutations induce an apparent DNA damage response in a disease-relevant setting of heterozygosity and cells carrying the mutations are not at a selective disadvantage in vivo. Using an assay that permits determination of telomere lengths from very small quantities of genomic DNA, the present study reveals telomere elongation upon prolonged presence of the heterozygous state of the Q623H mutation. For the Y223C mutation, elongation appears to occur more rapidly, although the first available time point is 71 days after targeting, leaving the kinetics of elongation unclear, while demonstrating a new telomere length that is significantly above WT.

The study addresses an important issue and is in my opinion well executed. As the heterozygous nature of the cells is of significance, the authors should make it clearer how the clonal identity of heterozygous cells was verified. Although clonal selection is mentioned in the methods, the Sanger sequencing of the Q623H / WT clone shown in EV 1 would also be consistent with Q623H / Q623H cells with minor WT contamination Unlike the sequencing trace shown for the heterozygous WT/MT clones following inaccurate repair in exon 9 (Fig 2E), the peaks corresponding to WT in Fig 1A middle panel are barely above background (Q623H/WT sample). It further appears that the banding patters in the STELA analysis shifts upwards for the later timepoints of the wt sample in EV2, although this is not seen in the gels shown in Fig 1E. This could be technical variation with the same genomic DNA input or it could be further indication of a mixed population of cells.
The authors should clarify whether the data plotted in Fig 1F is based on the bands in Fig 1E, or whether additional lanes were run and used in the analysis (such es those from EV2). The same question applies to Fig 4D and E). At least it appears to this reviewer that more datapoints are shown in the graphs than bands are visible on the blots.

Minor points:
It would help the reader if the nature of each mutant was mentioned in the figure or legend in addition to the main text. For example, in F2, EV3, 4 and 5 labels are sg-1; sgRNA-1, sgRNA, #P1-6 and #A1-5. A clearer reference to this all referring to specific exon 9 missense mutations would be helpful as done in Ev6 where the label Q214X is used and linked to sgRNA-2 in the text .

It should be explained that the abbreviations CFU-GM, CFU-GEMM and BFU-E refer to established morphologies for colony forming units of different hemopoietic lineages

Page 12, line 2 insert "per" in "was performed as _ manufacturer's protocol."

Referee #1:

Shelterin is a six-subunit protein complex that functions at telomeric DNA to ensure telomere end protection. It is well established that defects in telomere end protection induce both telomere dysfunction and changes in telomere length, phenotypes often associated with cancer progression. Next generation sequencing has identified mutations in the shelterin protein POT1 in several cancers including, chronic lymphocytic leukemia, familial Hodgkin Lymphoma, and familial melanoma. The mutations in POT1 are often heterozygous missense or nonsense mutations that are not accompanied by loss of heterozygosity. However, exactly how these mutations contribute to the development of cancer in these patients is unclear. Here, the authors aim to dissect the cancer-associated POT1 (caPOT1) mutations using a combination of human embryonic, and hematopoietic, stem cells to determine whether telomere dysfunction or telomere elongation (or both) contribute to tumorigenesis. Using a CRISPR genome editing strategy the authors have recreated a number of cancer-associated mutations in POT1 and demonstrated that telomere elongation, not telomere dysfunction, is likely the cellular phenotype that promotes tumorigenesis. Overall, the data are convincing and the story makes an important contribution to our understanding not only of telomere biology, but also how defects in telomere maintenance contribute to the development of cancer. There are a few comments/concerns provided below.

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1. The authors have attempted to recreate two known nonsense mutations in POT1 at position 214 and 250. The mutations that were generated are actually in/dels within exon 9. The authors argue that these in/dels resemble reported cancer-associated nonsense mutations at either amino acid 214 or 250. However, from the supplemental data the mutations appear to be around amino acid 226. Some numerical references would be helpful here to more precisely define the location of the in/dels. Combined, these in/dels have an allele frequency of about 25%, given that the 214 and 250 mutations are nonsense mutations, do these in/dels lead to any observable changes at the protein level? A WB for POT1 would be useful here.

Yes, the reviewer is correct that the sgRNAs used to make indels a generalization for the region. We screened several sgRNAs to identify those that will allow us to track indels in HSCs. We have now added a clearer description for this in the supplemental figure EV5C and modified the main text to explain this better. Specifically, EV5C now indicates some of the expected protein products generated by +/- 1nt indels at the sgRNA cut sites.

Unfortunately, we were not able to measure the protein levels of POT1 in the edited HSCs due to the limited number of cells we obtain from each step of the assay. The step we would get the largest number of cells is after colony-forming assay. However, the sizes of colonies grown in each well of 96 well plates range from several a few hundred cells to approximately a low few thousands of cells. This is not sufficient for us to perform a reliable WB analysis.

2. At what point was telomere deprotection analyzed in the Q623H/WT and Q623H/ Q623H mutant cell lines? Does telomere dysfunction accumulate as telomere length increases?

Thank you for pointing this out. The data shown were generated on day 94 after isolation this is now added to the legend. We have confirmed that the cells with the Q623H mutation remain TIF negative. It is important to note that long telomeres are not automatically associated with a DNA damage response. For example, *mus musculus* telomeres are much longer than human telomeres and yet fully protected and TIF negative. Similarly, RPE cells overexpressing TERT have elongated telomeres without TIFs. We now include a sentence about this in the discussion.

3. How did the caPOT1 mutations affect telomere length in the HSC population?

This is a really interesting and important question. We tried to address this, however as discussed above the limited material that we recover from the sorted bone marrow samples does not allow us to make any conclusive/significant statements about the change in telomere length. We were able to analyze some of the edited colonies after direct differentiation, but it did not show significant differences in telomere length between colonies. This might be because of the short period of time between the editing and the telomere length analysis. As we are not sure about the interpretation of this data we do not include it in the paper. Similarly, these experiments did not result in telomere length data for the experiments shown in figure 4.

4. The authors should include representative images of TIF in Figure 1b.

We thank the review for pointing this out. We now include this data, please see new Figure 1B.

5. For ease of comparison can Figure 2a and Figure 2d be graphed the same?

We made this change as suggested by the reviewer (new figure 4a and 4d).

6. In the text the authors state that they found two sgRNA that resulted in 20-25% gene editing. EV3 appears to demonstrate P1 and P2 were most efficient with nice data, however, the Figure legend for Figure 2a states that sgRNA-5 was used. sgRNA-1 is the label on the

axis in the graph itself. I assume reference to sgRNA-5 was a typo?

Yes, this was an inadvertent mistake. Thank you for pointing this out. We have corrected this now.

7. The authors state that they confirmed the results of the long-term xenograft experiments of POT1-edited cells using the Q214X caPOT1 nonsense mutation generated with sgRNA-2. The EV aren't entirely clear, are the in/dels actually leading to a specific nonsense mutation at 214? Or is this a generalization for that region? The authors should clarify this point.

The reviewer is correct that this is a generalization for the region. As also outlined above we had to screen several sg RNAs that resulted in efficient cutting in exon 9 of POT1. We have now added a clearer description for this in the supplemental figure EV2C and modified the main text.

8. Figure 1 and 5 should be combined or discussed sequentially. The flow seems a bit odd with them separated by other data given that they are addressing the same question?

We made the changes as suggested by the reviewer.

9. The authors conclude that telomere elongation drives the selection of caPOT1 mutations during cancer progression. However, this is overstated as the authors don't actually demonstrate cancer progression. The caPOT1 cells are not selected against and continue to contribute to the cellular population, but they aren't enriched over time and the authors have not analyzed tumorigenesis. The authors could refine the language here.

The reviewer raises an important point that our data does not directly address tumor progression. We have removed this speculation from the text.

10. Figure 4a is already published data and while useful for the reader to understand the data presented in Figure 4b, seems like 4a and Table 1 should be combined and put in the supplement? Or a note included under Figure 4a noting that this is previously published data (similar to Table 1). The authors do this in the text, but within the Figure itself is also warranted.

We would like to thank the reviewer for this suggestion. We feel that the pedigree (now Fig. 6A, former 5A) adds to a better understanding of the data shown in the telomere length blot (Fig. 6B, former 5B). We now add a comment directly in the figure panel to indicate that this data is published elsewhere.

Referee #3:

The role of POT1 mutations in various cancers is intriguing and worth exploring, and the question of whether POT1 mutations promote genome instability and thus facilitate tumorigenesis is not completely settled in the field. This study by Kim and co-authors presented their efforts in characterization of some cancer-associated POT1 (caPOT1) mutations in both human embryonic stem cells (hESCs) and hematopoietic stem cells (hCD34+) in a disease-relevant heterozygous setting. The authors claimed these caPOT1 mutants do not lead to telomere dysfunction-induced DDR, yet result in telomere lengthening, supporting the role of telomere lengthening in tumorigenesis. However, some caPOT1 mutants with these telomere phenotypes including A224A and S270A have been reported previously. In addition, other caPOT1 mutation, such as A532P, did not significantly lead to telomere lengthening. Thus, telomere lengthening may not be the sole basis of how POT1 mutations contributes to tumorigenesis. Nevertheless, it would be of great interests, if the authors explore (a) whether telomere lengthening is indeed in favor of tumorigenesis or not, for example, comparing the tumorigenesis potential of caPOT1 mutant cells with relatively short and long telomeres; and (b) how telomere lengthening contributes to tumorigenesis, for example, do caPOT1 mutant cells show any sign of replicative stress and genome instability? Without these additional experiments, their conclusion does not add novel mechanistic advancement to the topic. Some data interpretation and presentation also need further clarification (as follows).

1. That authors used hematopoietic stem cells (hCD34+) to test the effects of caPOT1 mutations is well accepted, given the close association of these mutations with hematopoietic tumors, such as CLL and lymphoma. However, the authors didn't justify the reason to use human embryonic stem cells (hESCs) as a cellular model. In fact, human stem cells respond to DNA damage very differently compared to somatic cells and lack certain cell cycle checkpoints (G1 and intra-S) ("Diminished apoptotic priming and ATM signaling confer a survival advantage onto aged hematopoietic stem cells in response to DNA damage". Nat Cell Biol. 2018 Apr; 20(4): 413-421"; "Attenuated DNA damage responses and increased apoptosis characterize human hematopoietic stem cells exposed to irradiation. Sci Rep 2018; 8, 6071"). In addition, a recent publication showed that uncapped hESCs exhibited a mutated damage response and can proliferate as wild type cells ("TRF2-mediated telomere protection is dispensable in pluripotent stem cells". Nature, Nov 25, 2020). Moreover, somatic cells with undetectable telomerase and limited telomere repeat reserve could hit the replicative senescence checkpoint, which acts as a tumor suppressor. Thus, telomere lengthening may help somatic cells overcome the replicative senescence checkpoint. In contrast, human stem cells express telomerase and have relatively longer telomeres. It is not

known if hESCs would hit the replicative senescence checkpoint in vivo. Thus, using stem cells to investigate POT1 mutations in regard to telomere-induced DDR and lengthening is not desirable. Somatic cell types may be considered, such as human melanocytes.

The reviewer raises some very relevant points. We chose to perform our experiment in hESCs because it allows us to analyze telomere DNA damage and telomere length changes in a genetically defined human system. Other primary cell systems such as human melanocytes, while highly desirable for such experiments, have not been established to allow for this type of analysis. Previous work in mouse cells is somewhat confounded by the dramatically long telomeres in *mus musculus*.

The reviewer's concern that hESCs might present a specialized model system with an altered DDR response as is well taken. However, it is important to point out that the work by the Denchi and Boulton groups describing that TRF2-mediated telomere protection is dispensable in pluripotent stem cells was done in mouse stem cells. These studies were performed under culture conditions that stabilize a naïve stem cell state. hESCs represent the primed state that recapitulates a pluripotent developmental state similar to the epiblast. It is clear from both papers that at this point of differentiation –the epiblast state– TRF2 is required, as TRF2 deficient embryos fail to develop. As for the other two papers the reviewer points out, it seems that these papers generally agree with our assessment that DDRs in hematopoietic stem cells results in cell death and strong counter selection. Our decision to study POT1 mutations in this system is motivated by attempting to study them in a disease relevant cell type where the DDR outcome has been well described. Hopefully, future experiments can address the role of caPOT1 mutations in melanocytes in which the cellular outcomes of the DDR responses have been less investigated.

2. One of the major conclusions of the manuscript is that caPOT1 mutants do not lead to telomere dysfunction-induced DDR, measured by TIFs. However, the authors only test the TIF formation in hESCs, but not in any other cells including hematopoietic stem cells (with mutations at positions 214 and 250? Not clear to me), or Cas9-edited POT1 cells after bone marrow isolation as in Fig 3, as well as patient peripheral lymphocytes with Y36H mutation as in Fig 4.

We agree with the reviewer that it would be great to analyze the Y36H in peripheral lymphocytes. The is only very limited material available and we have initiated the EBV transformation of these cells. These experiments however got delayed due to COVID-19. We are not sure if and when we will be able to perform these experiments. Thus, we feel that they are beyond the scope of this report. Yes, we attempted staining bone marrow sorted cells for TIFs, however these failed because of the low cell numbers.

3. The authors concluded that caPOT1 mutations lead to telomere elongation. However, throughout the manuscript only STELA is used to assess telomere length. STELA is a good method for single telomere length analysis, especially to detect a subpopulation of short telomeres, but lacking an overall measurement of bulk population of

telomere length. To firmly conclude on telomere elongation, Southern blot of telomere restriction fragment analysis (TRF analysis) is required to provide more information on telomere length (e. g. length heterogeneity, overhang change, elongation rate, etc.).

As suggested by the reviewer, we now include a revised panel EV2B that shows the TRF analysis for the Q623H cells.

4. In Fig 1, the authors stated in the texts that "... Y223C mutation showed a robust TIF response" and "All cells grow normally". First, growth curve of these cells compared to wild type cells should be included. Second, from Fig 1b, 2 POT1 Y223C/- hESCs showed 10-20% TIFs with high variation. Is this damage biologically meaningful? Does the "robust TIF response" cause any damage signaling and checkpoint activation?

Our current culture condition of the hESCs does not allow us to perform growth curves in the traditional way, as we are culturing our cells on MEFs. However, we have established that under these conditions, growth defects will become apparent when cells are cultured in parallel. Nevertheless, the reviewer's point is correct that our stem cells do not respond with an arrest phenotype and we agree that this is an interesting observation by itself. To ensure that these cells could form TIFs we analyzed the Y223C/- cells which also proliferate normally and infected the cells with TPP1DPBD to trigger TIFs. Our observations that TIF positive hESCs can proliferate is also supported independently by the TPP1 knockout in hESCs by the Songyang group (Kim H, Li F, He Q, Deng T, Xu J, Jin F, Coarfa C, Putluri N, Liu D, Songyang Z (2017)).

5. In Fig 3, the authors evaluated the long-term proliferative capacity of caPOT1 mutations in engrafted mouse bone marrow cells. After transplantation, edited cells should be tested for TIF formation, chromosome aberrations by metaphase, and telomere length by TRF analysis or qFISH at different time points to address whether there is progressive telomere elongation and genome instability, compare to unedited cells. Also, comparing the editing frequency of POT1 in hCD34+ cells before and after engraftment, it increased from 12% to 23%. Is this change significant, representing a positive selection advantage for cell proliferation? Finally, did the immune deficient mice after engraftment show any hematopoietic lineage change in differentiation pattern, and more importantly, any occurrence of hematopoietic neoplasia?

The reviewer raises a similar point as the reviewer 1 that we have not analyzed cancer progression in our experimental system. We agree our initial discussion was overstating in this regard and we have corrected this now. In fact, it seems important to note that POT1 heterozygous mice do not have a cancer phenotype and that only after homozygous activation of oncogenes or inactivation of tumor suppressors POT1 mutations have been reported to promote cancer formation. We have adjusted the text to better reflect this. To more directly

answer the reviewer's question: No, we did not observe any overt hematopoietic lineage change in differentiation pattern. We also did not see any overt occurrence of hematopoietic neoplasia. This is now noted in the manuscript.

6. In Fig 5c, the positive control of TPP1 delPBD only generated 5-20% TIF response, while in the published papers, dominant negative TPP1 delPBD alleles normally elicited at least 40-60% TIFs. Again, raising the question of "attenuated damage response" in stem cells.

We apologize for not explaining this experiment in more detail. The hESCs are infected with a lentivirus that expresses TPP1 DPBD without selection and analyzed 72 later. We include this control to counteract the concern raised by the reviewer in point 1. The fact that we can detect TIFs in these cells rules out that preexisting genetic changes or attenuation of an ATR response precludes TIF formation in these cells. We now explain this better in the materials and method section.

Minor points:

1. The Cas9-edited cells are clonal population. It is unclear in the manuscript where wt/wt cells used in STELA are pooled cells or clonal cells.
Thank you for pointing this out. We now are more explicit that all wt/wt cells are parallel unedited clones.

2. The authors introduced caPOT1 mutations into hematopoietic stem cells (hCD34+) at positions 214 and 250 without citing any reference or brief introduction of whether these two mutations have ever been characterized.

This point was also raised by the first reviewer. We now include a better explanation for this in the text and provide the reference for where these mutations have been published.

3. In Fig 5b, are wt/Y223C + TPP1 delPBD and wt/puro + TPP1 delPBD mislabeled?

We would like to thank the reviewer for catching this formatting mistake! This has been fixed now.

Referee #4:

The manuscript "Cancer-associated POT1 mutations lead to telomere elongation without induction of a DNA damage response" examines cancer-associated POT1 mutations in a physiologically relevant context to address the important question whether induction of a DDR and associated genome instability or telomere elongation underlies the cancer-promoting property of these mutations. Prior work has provided

support for both phenotypes based on overexpression studies. Given the exquisite importance of stoichiometries and absolute abundance of factors involved in telomere maintenance, a study that examines the effects of mutations in the context of genome-engineered heterozygosity was much needed and anticipated by the field. Using a combination of cell-based and long-term xenograft experiments, the authors demonstrate that none of the mutations induce an apparent DNA damage response in a disease-relevant setting of heterozygosity and cells carrying the mutations are not at a selective disadvantage in vivo. Using an assay that permits determination of telomere lengths from very small quantities of genomic DNA, the present study reveals telomere elongation upon prolonged presence of the heterozygous state of the Q623H mutation. For the Y223C mutation, elongation appears to occur more rapidly, although the first available time point is 71 days after targeting, leaving the kinetics of elongation unclear, while demonstrating a new telomere length that is significantly above WT.

The study addresses an important issue and is in my opinion well executed. As the heterozygous nature of the cells is of significance, the authors should make it clearer how the clonal identity of heterozygous cells was verified. Although clonal selection is mentioned in the methods, the Sanger sequencing of the Q623H / WT clone shown in EV 1 would also be consistent with Q623H / Q623H cells with minor WT contamination Unlike the sequencing trace shown for the heterozygous WT/MT clones following inaccurate repair in exon 9 (Fig 2E), the peaks corresponding to WT in Fig 1A middle panel are barely above background (Q623H/WT sample). It further appears that the banding patters in the STELA analysis shifts upwards for the later timepoints of the wt sample in EV2, although this is not seen in the gels shown in Fig 1E. This could be technical variation with the same genomic DNA input or it could be further indication of a mixed population of cells. The authors should clarify whether the data plotted in Fig 1F is based on the bands in Fig 1E, or whether additional lanes were run and used in the analysis (such es those from EV2). The same question applies to Fig 4D and E). At least it appears to this reviewer that more data points are shown in the graphs than bands are visible on the blots.

The reviewer raises an important point about the clonality of our stem cells. We apologize that we failed to mention in the text that we ensured the accuracy of our genotyping and ruled out the expansion of a subpopulation we genotyped our cells at the end of the telomere length experiments. We also subcloned the genotyping PCR amplicons from the Q623H/WT single cell derived clones into PCR2.1 to confirm our genotyping results. We now specifically mention this analysis in the material and methods and include the subcloning data in the EV1.

Regarding the STELA, we now more indicate the data points that are used in the quantifications. The same genomic DNA from each cell line was used to run multiple STELA

experiments, and the quantification shown in figure 1F (now Figure 2A) includes two separate experiments that were analyzed independently and represented on the same graph. The additional STELA blot can be found in EV2C.

Minor points:
It would help the reader if the nature of each mutant was mentioned in the figure or legend in addition to the main text. For example, in F2, EV3, 4 and 5 labels are sg-1; sgRNA-1, sgRNA, #P1-6 and #A1-5. A clearer reference to this all referring to specific exon 9 missense mutations would be helpful as done in Ev6 where the label Q214X is used and linked to sgRNA-2 in the text .

We apologize for this. We now have unified the sg-RNA nomenclature.

It should be explained that the abbreviations CFU-GM, CFU-GEMM and BFU-E refer to established morphologies for colony forming units of different hemopoietic lineages

Changed as suggested by the reviewer.

Page 12, line 2 insert "per" in "was performed as _ manufacturer's protocol."

Thank you! We reworded this.

Additional changes:

We apologize for inadvertently not referencing also the recent work by Pinzaru. We now include a reference to their recent overexpression studies of caPOT1 cancer alleles in the introduction of our paper. It is important to note that our studies are complementary with this work. While Pinzaru and colleagues describe the phenotypes with POT1 overexpression in telomerase positive cancer cells, their work does not focus on addressing the phenotypes associated with heterozygous POT1 mutations.

1st Revision - Editorial Decision 1st Mar 2021

Thank you for submitting your revised manuscript to The EMBO Journal. It has now been re-reviewed by referees 1 and 3, who found the original concerns satisfactorily addressed and have now further reservations regarding publication. We shall therefore be happy to accept the study for The EMBO Journal, following incorporation of some remaining editorial issues, as listed below.

REFEREE REPORTS

Referee #1:

The authors have addressed my concerns and I support publication.

Referee #3:

The authors have addressed majority of my questions. The revised manuscript was largely improved. Nevertheless, the key questions remain unclear whether telomere lengthening is indeed in favor of tumorigenesis, and how telomere lengthening contributes to tumorigenesis, which may be beyond the scope of this study.

2nd Authors' Response to Reviewers 10th Mar 2021

The authors have made all requested editorial changes.

Accepted **16th Mar 2021**

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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Dirk Hockemeyer
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-107346

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg 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/varied/perturbed in a controlled manner.

- 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 shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

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http://datadryad.org

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http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We performed the exoeirments as indicated in the material and methods. We chose to test two sgRNAs for the in vivo engrafment. We indicate animal numbers in the EV
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We report all data for the conengrafted experiments performed in
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Does not apply, as alleles frequencies are followed within the same animal.
For animal studies, include a statement about randomization even if no randomization was used.	We did not randomize.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	TIF were scored blinded.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal studies were not blinded
5. For every figure, are statistical tests justified as appropriate?	Yes we apply, statistical test that are the standart in the field and used for these experiments.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Does not apply.

Is the variance similar between the groups that are being statistically compared?	Does not apply.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Boyle. MCOB 2020.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Lengner et al.. NIH stem cell registry# 0079. We monthly test for mycoplasma.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Animals were housed in the LKS anaimal facility, UC Berkeley. Male and femal NBSGW mice betweeen 6 and 12 wweks of age were used.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	AUP-2015-10-8041
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes.
12. 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.	Yes
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	does not apply
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NIH guidelines should be applied.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	does not apply.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	does not apply
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	does not apply

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	does not apply.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	does not apply.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	does not apply.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	does not apply.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Does not apply