THE EMBO JOURNAL

Human Shelterin Protein POT1 Prevents Severe Telomere Instability Induced by Homology-Directed DNA Repair

Galina Glousker, Anna-Sophia Briod, Manfredo Quadroni, and Joachim Lingner
DOI: 10.15252/embj.2020104500

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Review Timeline:

Submission Date:	17th Jan 20
Editorial Decision:	21st Feb 20
Revision Received:	20th Aug 20
Editorial Decision:	10th Sep 20
Revision Received:	15th Sep 20
Accepted:	18th Sep 20

Editor: Hartmut Vodermaier

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. 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 characterization of POT1 loss-of-function consequences in human cells for our consideration. It has now been assessed by three expert referees, whose comments are copied below for your information. While referees 1 and 2 are in principle supportive of publication pending adequate revisions, referee 3 is not convinced that the study provides sufficient new insight over previous work. Following further consultations with the referees, emphasizing the (patho)physiological importance of characterizing human POT1 and its recombination-related roles that do not seem to be shared by murine homologues, we have decided to consider the study further for eventual EMBO Journal publication, provided that you can satisfactorily address the various experimental/specific issues raised in the reports. The most important point here will be to validate key findings in more than one cell line/clone as requested by referees 1 and 3 - given the prevalent confusion about POT1 function based on the mouse gene deletion and human knockdown studies reported so far, I do think it would be essential to now decisively clarify that the new findings on human POT1 function are generally relevant irrespective of cell line background etc. Should this necessitate an extended revision period, please do not hesitate to contact me, also if you should have any other questions/comments regarding the referee reports or this decision. As usual, competing manuscripts published elsewhere during your revision will not affect our final decision on your study, but our single-revision-round policy at the same time makes it important to comprehensively answer to all points raised at this stage.

Further information on preparing 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 - I look forward to your revision.

Referee #1:

In the manuscript "Human POT1 prevents severe telomere instability induced by homology directed DNA repair", the authors discover an intriguing novel role for POT1 in the suppression of homology-directed repair pathways at telomeres. The data are not entirely unexpected. It seems reasonable that if you remove the major ss telomeric binding protein, other ss DNA binding and repair proteins

(including HDR pathways) will be required to step in, and there is some evidence in the literature to suggest that this will happen. Nevertheless, this has not been definitively shown. The study is entirely conducted in modified HEK293E cells, which first contain tagged TRF1 and TRF2 genes (for the proteomic analysis), and second contain conditional POT1 knockout alleles. The authors demonstrate that POT1 loss results in telomere dysfunction, as well as telomere elongation and the accumulation of ss G-rich telomeric DNA. This is accompanied by apoptosis. The authors then use a proteomic approach to characterise the telomeric proteome in cells lacking POT1, and identify enrichment of a number of pathways, including HR proteins. The significance of the HR pathway to the POT1 KO phenotype is demonstrated by suppression of telomere extension when various HR factors are depleted in the POT1 KO background. The authors finally demonstrate the induction of some ALT-associated phenotypes with POT1 loss, but are notably cautious not to say there is induction of ALT.
Overall, this is a straightforward paper. I have a few suggestions to improve/strengthen the findings.
1. One major concern is that this has only been done in one cell line (HEK293E), which has been substantially manipulated. Demonstration of the striking telomere lengthening and ss G-rich strand accumulation in other cell lines would really strengthen the findings.
2. What are the "parental EV" and "parental POT1 gRNA" controls in Fig 1C?
3. Fig S2B would benefit from some arrows to indicate colocalizations.
4. I'm not sure about the terminology "fuzzy" or "split into two" to describe fragile telomeres (p8). Could this be changed.
5. In Fig 3D, I am confused by lanes 1-5. Why are lanes 1 and 3 different, when the treatments are the same, ie. you seem to get rescue in lane 3 when this only contains EV? In addition, both lanes 4 and 5 appear to have rescue by 3HA-POT1, when only lane 5 has Dox.
6. Was the QTIP done in just one clone? Could this be clarified.
7. I would like to see further characterization of the telomere elongation. EdU incorporation followed by pull-down/dot blot or IF/FISH would be a nice inclusion to show that the DNA is newly synthesised (as has been done in several recent studies, including from the Greenberg lab), or the recently described ATSA assay (Zhang et al., 2019).
8. Suppression of telomere elongation and ss G-rich strand accumulation is most modest with BLM kd, while BLM seems to be the most critical component for telomeric HDR. Can the authors offer some discussion regarding this finding? Does POLD3 kd impact the POT1 KO telomeric DNA phenotype?
9. The labelling of the TRF gels is inconsistent eg between Fig 3 and Fig 6.
10. I would prefer to see telomeric damage by IF/FISH rather than global damage by WB following HDR inhibition (Figs S5A and S5B).
11. Is any change in C-rich telomeric DNA detected? Have native/denatured TRFs probed for the C-rich strand been done?
12. The manuscript would benefit from proof reading and consistency in nomenclature.
13. I like the model presented in Fig 7.

Referee #2:

The six subunit shelterin complex is important for end protection for preventing chromosome ends from being recognized as a double-strand break. The specific function of all 6 components in human cells is not fully understood. Here, the authors show that deletion of the single-stranded DNA binding subunit, POT1 in a human cells leads to formation of aberrant structures at telomeres, increased HR, and increases in telomere length most likely through recombination. Furthermore, the authors use a novel 2-step QTIP approach to outline the alterations in the telomeric proteome

upon POT1 depletion.

So far, the function of POT1 has been primarily studied using partial inhibition in human cells or via gene deletion of the two POT1 paralogs in mouse cells. Both approaches have their inherent flaws and have been over interpreted. In this nice study linger and his team establishes, once and for all, the function of POT1 in human cells by clean genetic KO studies. The work is extensive and rigorous. This is an important study that will have significant implication in the telomere field and provides insight into the function of mutant POT1 in tumors.

Minor points:

Figure 2
The specific criteria used to identify fragile ends and fusions should be clarified in the methods section. A separate question: do the changes in telomere length / signal intensity due to POT1 depletion affect the frequency of telomere fragility. Can the authors use a better negative control with matching telomere length?

Figure 3
How do the phenotypes presented for the KO cells compare when POT1 OB mutant is overexpressed in wiltype cells? For example, does the OB also lead to fragile sites and increased HR?

Referee #3:

In this manuscript the authors set out to analyze human POT1 function using CRISPR gene editing and proteomic analysis in HEK293 cells. The authors demonstrate that loss of POT1 leads to a number of hallmarks of telomere dysfunction including an increase in telomere length heterogeneity, branched telomeric DNA structures, telomere fragility, and telomeric R-loop formation. Given these phenotypes, the authors looked to further define the telomeric proteome following POT1 knockout using an improved telomeric chromatin isolation protocol originally pioneered in the Lingner lab. Following Q-TIP, the authors identified 88 proteins that were enriched at telomeres following POT1 knockout by more than 1.5-fold as compared to control cells. These proteins are known to regulate cell cycle and mitosis, DNA damage response and repair, and RNA metabolism. The authors use this data to further support the main conclusion of the manuscript, that POT1 functions to suppress homology directed repair at telomeric DNA. Although much of the current study has been previously published in two independent papers, Hockemeyer et al 2006 and Wu et al 2006, Glousker et al argue that the knockout of POT1 in human cells allows them to demonstrate phenotypes that had not been anticipated from the previous studies that relied on conditional depletion. However, the main conclusions of this manuscript do not extend beyond what has already been published in those two previous manuscripts limiting the novelty of the study and enthusiasm for publication.

Major Comments
• Hockemeyer 2006 and Wu 2006 use conditional deletion of POT1a and POT1b in MEFs and demonstrate a significant increase in chromosome fusion events following deletion of POT1. In addition, in the Hockemeyer manuscript Figure 6 and Wu manuscript Figures 5 and 6 demonstrate that loss of POT1 leads to changes in telomere length consistent with the results presented here

by Glousker, including rapid induction of telomere elongation (within several days), branched telomeric structures, and aberrant telomere homologous recombination. The major difference is that the POT1 KO MEFs from the 2006 papers undergo senescence, while the fate of the POT1 KO HEK293 cells here were not clearly described. Glousker et al. highlight that the telomere elongation events that they observe are independent of telomerase suggesting they undergo some sort of homology-directed repair. However, both telomere elongation and the telomerase independent nature of the phenotype have already been shown in Hockemeyer et al 2006 and Wu et al 2006.

• Glousker et al go on to highlight changes in the proteome following POT1 knockout using QTIP, but the changes in the proteome are modest, not validated by any other method, nor are they entirely novel as complexes like MRN have been previously demonstrated to regulate DNA damage repair at telomeres following POT1 loss (Wu et al 2006).

• The experiments in this study also originate from one single clone from a single cell line (HEK293E). This is not sufficient to reflect a general phenomenon which may not be consistent in additional cell lines with varying genetic backgrounds.

Re: EMBOJ-2020-104500
Human POT1 Prevents Severe Telomere Instability Induced by Homology Directed DNA Repair

Referee #1:

In the manuscript "Human POT1 prevents severe telomere instability induced by homology directed DNA repair", the authors discover an intriguing novel role for POT1 in the suppression of homology-directed repair pathways at telomeres. The data are not entirely unexpected. It seems reasonable that if you remove the major ss telomeric binding protein, other ss DNA binding and repair proteins (including HDR pathways) will be required to step in, and there is some evidence in the literature to suggest that this will happen. Nevertheless, this has not been definitively shown. The study is entirely conducted in modified HEK293E cells, which first contain tagged TRF1 and TRF2 genes (for the proteomic analysis), and second contain conditional POT1 knockout alleles. The authors demonstrate that POT1 loss results in telomere dysfunction, as well as telomere elongation and the accumulation of ss G-rich telomeric DNA. This is accompanied by apoptosis. The authors then use a proteomic approach to characterise the telomeric proteome in cells lacking POT1, and identify enrichment of a number of pathways, including HR proteins. The significance of the HR pathway to the POT1 KO phenotype is demonstrated by suppression of telomere extension when various HR factors are depleted in the POT1 KO background. The authors finally demonstrate the induction of some ALT-associated phenotypes with POT1 loss, but are notably cautious not to say there is induction of ALT.
Overall, this is a straightforward paper. I have a few suggestions to improve/strengthen the findings.

1. One major concern is that this has only been done in one cell line (HEK293E), which has been substantially manipulated. Demonstration of the striking telomere lengthening and ss G-rich strand accumulation in other cell lines would really strengthen the findings.

Response: We have managed to reproduce the POT1 KO phenotype in HeLa cells using co-transfection of the cells with 2 gRNAs targeting *POT1* (Fig. EV1). Interestingly, knockdown of POT1 with shRNA to the level of complete disappearance of the POT1 signal on the western blot did not induce neither the telomere lengthening nor G-rich strand accumulation in HeLa cells (Fig. EV2). Therefore, the very small amounts of residual POT1 at telomeres in case of shRNA-mediated knockdown are enough to suppress recombination at telomeres. A complete disappearance of POT1 is required to reveal its crucial functions in suppressing recombination.

2. What are the "parental EV" and "parental POT1 gRNA" controls in Fig 1C?

Response: This refers to the parental cell line 293E_cl75 that was used for generation of inducible *POT1* KO cell lines. We modified the labeling on the Fig1 C and clarified it in the figure legend.

3. Fig S2B would benefit from some arrows to indicate colocalizations.

Response: The *POT1* KO leads to a very strong DNA damage response, hence, a majority of telomeres co-localize with γH2AX foci. Yet, we have added the arrows, but specified that they only indicate a subset of colocalizations.

4. I'm not sure about the terminology "fuzzy" or "split into two" to describe fragile telomeres (p8). Could this be changed.

Response: We changed “fuzzy” and "split into two" to “smeary” and “double” respectively, which are attributes that have been commonly used in the field to describe telomere fragility.

5. In Fig 3D, I am confused by lanes 1-5. Why are lanes 1 and 3 different, when the treatments are the same, ie. you seem to get rescue in lane 3 when this only contains EV? In addition, both lanes 4 and 5 appear to have rescue by 3HA-POT1, when only lane 5 has Dox.

Response: Thank you for noting this error. We have corrected the labelling. As for lanes 4 and 5, the labeling is correct, and there is no rescue in lane 4, but it unfortunately contains less DNA. In lane 5, there is a rescue.

6. Was the QTIP done in just one clone? Could this be clarified.

Response: We clarified this in the text (p12).

7. I would like to see further characterization of the telomere elongation. EdU incorporation followed by pull-down/dot blot or IF/FISH would be a nice inclusion to show that the DNA is newly synthesised (as has been done

in several recent studies, including from the Greenberg lab), or the recently described ATSA assay (Zhang et al., 2019).

Response: Unfortunately, due to technical problems we were not able to perform these experiments, but other data in the paper confirm that telomeric DNA upon *POT1* KO is newly synthesized: first of all, if the elongation was only due to the recombination of the existing DNA, we would have seen in addition to drastic telomere elongation, a drastic telomere loss on metaphase spreads and TRF gels, which we do not observe (Fig 2). Second, we now demonstrate that the phenotype we observe is at least partially dependent on the DNA polymerase subunit POLD3 supporting the notion that telomere elongation depends on DNA synthesis (Fig. EV2).

8. Suppression of telomere elongation and ss G-rich strand accumulation is most modest with BLM kd, while BLM seems to be the most critical component for telomeric HDR. Can the authors offer some discussion regarding this finding? Does POLD3 kd impact the POT1 KO telomeric DNA phenotype?

Response: We have modified the text. We have also demonstrated that in case of BLM knockdown the telomeric recombination is partially, but not fully suppressed, using the C-circle assay (Fig EV1). The only partial suppression of the phenotype upon BLM knockdown may be due to the residual amounts of BLM that were not depleted.

We now tested if depletion of POLD3 prevents telomere elongation upon POT1 loss. We were able to reduce POLD3 mRNA levels to approximately 20% and observed that under these circumstances, telomere elongation upon *POT1* deletion was significantly reduced (Fig EV2). Therefore, POLD3 contributes to telomere elongation though we cannot exclude that other polymerase are involved.

9. The labelling of the TRF gels is inconsistent eg between Fig 3 and Fig 6.

Response: Inconsistencies in the labeling are due to different types of gels (constant-field electrophoresis vs pulsed-field electrophoresis) and experimental setups. This is now clarified in the legends of the corresponding figures.

10. I would prefer to see telomeric damage by IF/FISH rather than global damage by WB following HDR inhibition (Figs S5A and S5B).

Response: We have now done this quantifying telomere dysfunction induced foci (TIFs) (see Fig S6B).

11. Is any change in C-rich telomeric DNA detected? Have native/denatured TRFs probed for the C-rich strand been done?

Response: We have done this experiment and we see no change in C-rich telomeric DNA on the TRF gels (Fig. 6E). However, we do detect an increase in C-circles upon POT1 KO.

12. The manuscript would benefit from proof reading and consistency in nomenclature.

Response: We have proofread the manuscript and tried to eliminate all errors.

13. I like the model presented in Fig 7.

Response: We are grateful for this comment.

Referee #2:
The six subunit shelterin complex is important for end protection for preventing chromosome ends from being recognized as a double-strand break. The specific function of all 6 components in human cells is not fully understood. Here, the authors show that deletion of the single-stranded DNA binding subunit, POT1 in a human cells leads to formation of aberrant structures at telomeres, increased HR, and increases in telomere length most likely through recombination. Furthermore, the authors use a novel 2-step QTIP approach to outline the alterations in the telomeric proteome upon POT1 depletion.

So far, the function of POT1 has been primarily studied using partial inhibition in human cells or via gene deletion of the two POT1 paralogs in mouse cells. Both approaches have their inherent flaws and have been over interpreted. In this nice study linger and his team establishes, once and for all, the function of POT1 in human cells by clean genetic KO studies. The work is extensive and rigorous. This is an important study that will have

significant implication in the telomere field and provides insight into the function of mutant POT1 in tumors.

Minor points:

Figure 2
The specific criteria used to identify fragile ends and fusions should be clarified in the methods section. A separate question: do the changes in telomere length / signal intensity due to POT1 depletion affect the frequency of telomere fragility. Can the authors use a better negative control with matching telomere length?

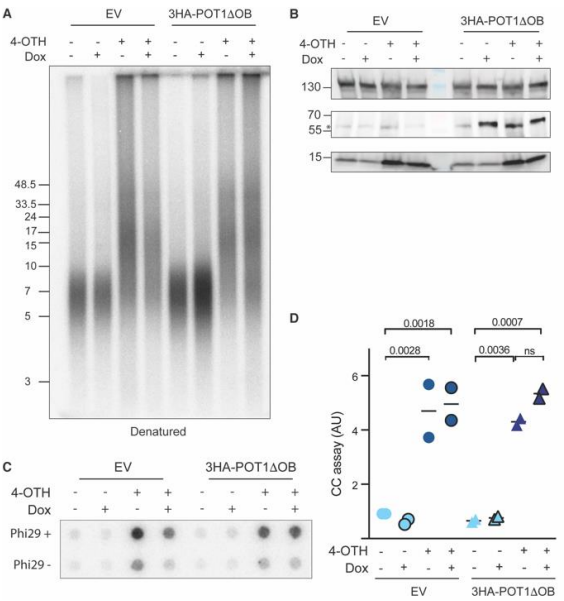
Response: We have now added a better description in the methods section how we scored telomere fragility.

To control for the telomere elongation, we used the cells that have been treated with 4-OHT for 4 days. These cells have the same telomere length as the untreated cells, but have already lost POT1 completely (Fig. S3A, B). As one can see from Fig 2D, there is a significant increase in telomere fragility already at day 4 of 4-OHT treatment.

In addition, we have looked at telomere fragility in clone 38, that has longer telomeres than clone 35 (Fig. S3C, D) and we see that despite having longer telomeres the percentage of fragile telomeres is lower in uninduced clone 38. However, on day 7 of treatment with 4-OHT the percentage of fragile telomeres is doubled, recapitulating the results we obtained with clone 35.

Figure 3
How do the phenotypes presented for the KO cells compare when POT1 OB mutant is overexpressed in wiltype cells? For example, does the OB also lead to fragile sites and increased HR?

Response: In our experimental setup overexpression of the POT1 OB mutant in wildtype HEK293E cells for 4 days did not lead to any change in telomere length (see Figure below). We also did not observe an increase of HR (based on the C-circle assay, see Figure below).



Referee #3:

In this manuscript the authors set out to analyze human POT1 function using CRISPR gene editing and proteomic analysis in HEK293 cells. The authors demonstrate that loss of POT1 leads to a number of hallmarks of telomere dysfunction including an increase in telomere length heterogeneity, branched telomeric DNA structures, telomere fragility, and telomeric R-loop formation. Given these phenotypes, the authors looked to further define the telomeric proteome following POT1 knockout using an improved telomeric chromatin isolation protocol originally pioneered in the Lingner lab. Following Q-TIP, the authors identified 88 proteins that were enriched at telomeres following POT1 knockout by more than 1.5-fold as compared to control cells. These proteins are known to regulate cell cycle and mitosis, DNA damage response and repair, and RNA metabolism. The authors use this data to further support the main conclusion of the manuscript, that POT1 functions to suppress homology directed repair at telomeric DNA. Although much of the current study has been previously published in two independent papers, Hockemeyer et al 2006 and Wu et al 2006, Glousker et al argue that the knockout of POT1 in human cells allows them to demonstrate phenotypes that had not been anticipated from the

previous studies that relied on conditional depletion. However, the main conclusions of this manuscript do not extend beyond what has already been published in those two previous manuscripts limiting the novelty of the study and enthusiasm for publication.

Response: We disagree with the above statements. The Hockemeyer et al 2006 and Wu et al 2006 papers are on the mouse Pot1 genes. As discussed in our and the cited papers they have considerably diverged from human POT1 in structure and function. Most significantly and as stated in the Hockemeyer abstract, “mouse telomeres require two distinct POT1 proteins whereas human telomeres have one. Such divergence is unprecedented in mammalian chromosome biology and has implications for modeling human telomere biology in mice.” Thus, the mouse work does not allow conclusions to be drawn for human POT1. Furthermore, in the Hockemeyer paper, mouse Pot1 was not seen to suppress homologous recombination at telomeres. In Wu et al., Pot1a deficiency caused telomere elongation and increased sister chromatid exchange. However, a role of RAD51, POLD3, BLM and others was not demonstrated.

Major Comments

• Hockemeyer 2006 and Wu 2006 use conditional deletion of POT1a and POT1b in MEFs and demonstrate a significant increase in chromosome fusion events following deletion of POT1. In addition, in the Hockemeyer manuscript Figure 6 and Wu manuscript Figures 5 and 6 demonstrate that loss of POT1 leads to changes in telomere length consistent with the results presented here by Glousker, including rapid induction of telomere elongation (within several days), branched telomeric structures, and aberrant telomere homologous recombination. The major difference is that the POT1 KO MEFs from the 2006 papers undergo senescence, while the fate of the POT1 KO HEK293 cells here were not clearly described. Glousker et al. highlight that the telomere elongation events that they observe are independent of telomerase suggesting they undergo some sort of homology-directed repair. However, both telomere elongation and the telomerase independent nature of the phenotype have already been shown in Hockemeyer et al 2006 and Wu et al 2006.

Response:

- As explained above, the Hockemeyer and Wu 2006 papers are on mouse Pot1 genes and not on human POT1.
- The two papers disagree with their conclusions.
- The mechanism of the telomerase-independent telomere processing step in POT1b deficient cells (Hockemeyer et al 2006) was not elucidated. We show here that increased 3’ overhangs in POT1-deficient human cells are RAD51-dependent.
- In the Hockemeyer et al 2005 paper where POT1 was knocked-down in human cells the here-described phenotypes were not observed. We see in our study that only upon complete loss of POT1 upon deletion of the gene the suppression of homologous recombination at telomeres is lost. Specifically, when we depleted POT1 in HeLa with shRNAs as done by Hockemeyer, telomere elongation is not seen (Figure EV4). Only upon complete KO of POT1, the telomere recombination phenotype unravels (Figure EV3).
- In Wu et al 2006 increased recombination is described in case of mouse Pot1a deficiency, not Pot1b but again these phenotypes where not reported in the Hockemeyer 2006 paper.

• Glousker et al go on to highlight changes in the proteome following POT1 knockout using QTIP, but the changes in the proteome are modest, not validated by any other method, nor are they entirely novel as complexes like MRN have been previously demonstrated to regulate DNA damage repair at telomeres following POT1 loss (Wu et al 2006).

Response:

- The referee acknowledges in the introductory paragraph that we detected 88 proteins that changed in abundance at telomeres upon POT1 KO. MRN is one of them and can be viewed as a positive control. For most others, it would be fair to state that they provide a tremendously rich resource.

- We demonstrate that the KD of several of the identified proteins that associate with POT1-depleted telomeres suppresses the POT1 KO phenotype – In our view this provides excellent validation.

- We identify more than 80 proteins that are present at telomeres upon POT1 loss in human cells.
- Roles of BLM, BRCA1, BARD1, RAD51 in cells lacking POT1 have not been previously described.

• The experiments in this study also originate from one single clone from a single cell line (HEK293E). This is not sufficient to reflect a general phenomenon which may not be consistent in additional cell lines with varying genetic backgrounds.

Response: We observed the phenotype we describe in several clones derived from HEK293E (Appendix Fig S1D, E, S3C). Moreover, we managed to reproduce this phenotype in HeLa cells (Fig EV3).

Thank you for submitting your revised manuscript to The EMBO Journal. Two of the reviewers have now looked at it again, and as you will see below, consider key issues of the study significantly improved in response to the original comments. Especially in light of the important extension to a second human cell line, we would therefore be happy to proceed further with publication of the study, following a final round of minor revision to address several remaining specific concerns by referee 3. In addition to incorporating these comments and suggestions into the final manuscript, please also again provide a point-by-point response answering to this report.

In addition, please also address the following editorial points during this final modification:

Referee #1:

The authors have addressed all my concerns. I am particularly happy with the extension of the investigations to include POT1 KO in a second human cell line (HeLa). Evaluation of HeLa POT1 KO cells demonstrated similar telomere phenotypes to those seen in 293E cells, and also identified that shRNA-mediated depletion is insufficient to induce the telomere phenotypes seen with complete KO. I feel the manuscript is suitable for publication in EMBO J.

Referee #3:

In the revised manuscript Glousker et al. have provided some additional data to support their finding that loss of POT1 leads to a number of hallmarks of telomere dysfunction including an increase in telomere length heterogeneity, branched telomeric DNA structures, telomere fragility, and telomeric R-loop formation. Most important in the revised manuscript was the generation of an additional POT1 knockout cell line to recapitulate the initial findings that had initially only been presented in HEK293E cells. There is no debate that the there is a divergence in the POT1 gene between human and mice. POT1a and POT1b clearly have distinct functions and these functions are largely conserved in the single human POT1 orthologue. Likewise, the data presented on the human gene in this manuscript are not unanticipated based on the overarching conclusions of previous publications analyzing both human and mouse POT1 genes. However, Glousker et al. suggest that severe telomere maintenance defects had 'not been anticipated' from previous depletion studies. This is simply overstated. At the very least, the language in the manuscript should be edited accordingly. In addition, there a few points below that should be addressed.

1. In Figure 1E the authors argue that after 4 days loss of POT1 leads to an increase in the subG1 population indicative of cell death via apoptosis. The FACS profiles in Figure S2A are from few cells overall and the sub-G1 population itself represents a small percentage. The data in Figure 1E demonstrate that the bulk of the cells undergo an arrest in growth, but not a decrease in PD that would indicate cell death. The authors should either clearly demonstrate the consequences of POT1 loss on cellular growth and/or survival. Does loss of POT1 lead to death, senescence, or growth arrest or modify the text to more accurately reflect the data presented.

2. The manuscript states that 157 proteins were significantly enriched at telomeres that lacked POT1 at day 7, however the table presented in EV1 has 150? Sheet TMT final (M6) column V 'T-test Significant, NT_D7:BH005'.

3. The authors have not validated the QTIP data for enrichment of these proteins at telomeres. Although the KD of these proteins to some extent suppress the POT1 KO phenotype, this data doesn't directly suggest the enrichment of these proteins at POT1 depleted telomeres which is the conclusion from these experiments.

4. In Figure 3 the tamoxifen abbreviation has a typo, it should be 4OHT not 4OTH.

5. It's not clear how the R-loops fit into the model how do the authors think about the increase in R-loops following POT1 deletion and can this be better described in the manuscript?

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Response: We are grateful for the comments of this referee that helped us improve our study.

Referee #3:

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Response: We now specify in the abstract that the “severe telomere maintenance defects that had not been anticipated from previous depletion studies in human cells.” Our data clearly unravel novel functions for human POT1. The phenotypes we observe in this work had not been described before and can only be observed upon complete loss of POT1. Therefore, they were not anticipated. Our work demonstrates that RNA interference-mediated depletion of POT1 is not effective enough to unravel the newly discovered functions of human POT1.

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Response: We agree that FACS profiles in figure S2A are from not so many cells due to technical issues. However, this experiment has been done in triplicate and the results are consistent between the biological replicates. From the cell cycle profiles it is obvious that fraction of cells in sub-G1 is increasing from 4% in NT cells to more than 20% in POT1 KO cells which is very substantial. Consistently, population of cells in G1 is decreased from 43% in NT cells to 23% in POT1 KO cells. This and the data shown in the Fig 2E indicate that the cells are dying during or shortly after M phase as a result of genomic instability and chromosome bridges between the daughter cells.
In the early stages of the study we have performed β-galactosidase staining and did not observe the increase in cells in senescence. The data in Fig 1E is representing the time frame of the experiment. When we keep growing the cells, even in the presence of 4-OHT, eventually POT1 KO cells are outgrown by a minor fraction of cells in which Cre-mediated recombination inducing POT1 KO was not efficient.

2. The manuscript states that 157 proteins were significantly enriched at telomeres that lacked POT1 at day 7, however the table presented in EV1 has 150? Sheet TMT final (M6) column V 'T-test Significant, NT_D7:BH005'.

Response: We are grateful for this comment, it was indeed a mistake and we now have corrected it in the manuscript.

3. The authors have not validated the QTIP data for enrichment of these proteins at telomeres. Although the KD of these proteins to some extent suppress the POT1 KO phenotype, this data doesn't directly suggest the enrichment of these proteins at POT1 depleted telomeres which is the conclusion from these experiments.

Response: Depletion of RAD51 and BRCA2 fully rescued the detrimental recombination at telomeres (Fig 6). Depletion of BRCA1, BARD1 and BLM are indeed rescuing the phenotype only partially, but as we refer to it in the text of the manuscript, it can be explained by incomplete depletion of the proteins with siRNAs. These results provide excellent evidence that these HR proteins not only associate with telomeres upon POT1 loss but they also cause detrimental recombination events. Our experimental design includes identification of the proteins at telomeres in WT and POT1 conditions, in three biological replicates. We analyze proteins that are statistically significantly enriched at telomeres. Moreover, presence of RAD51 at telomeres has recently been shown in two

additional studies from our laboratory (Briod et al, 2020, Feretzaki et al, 2020).

4. In Figure 3 the tamoxifen abbreviation has a typo, it should be 4OHT not 4OTH.

Response: We are grateful for this comment, it was indeed a mistake and we now have corrected it on the figure.

5. It's not clear how the R-loops fit into the model how do the authors think about the increase in R-loops following POT1 deletion and can this be better described in the manuscript?

Response: We address this in the manuscript, on page 21 and in the legend for Figure 7.

2nd Revision - Editorial Decision 18th Sep 2020

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: Joachim Lingner
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-104500

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 χ2 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.

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?	Sample size was determined empirically and based on the common pracitce in the field (literature search) , no statistical test was used to predetermine the sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	All the microscopy experiments were performed in three replicates in the blinded fashion.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	According to the literacture in the field analysis of telomeric dysfunction induced foci, PML foci and telomere aberrations assume normal distribution.
Is there an estimate of variation within each group of data?	No

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http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	The variance is similar in the datasets used in the manuscript

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).	Primary antibodies were anti-ATM pS1981 (1:1,000, ab81292; Abcam), anti-γH2AX (1:1,000, 05-636; Millipore), anti- CHK1-pS345 (1:1000, #2348, Cell signaling), anti-CHK1 (1:1000, sc-8408, Santa-Cruz), anti-RPA32 –S33 (1:1000, A300-246A, Bethyl), anti-vinculin (1:3000, ab129002, Abcam), anti-POT1 (1:1000, ab124784, Abcam) and anti-HA (1:3000, BLG-901502, BioLegend). Secondary antibodies were HRP-conjugated goat anti-mouse (W4021, Promega) and goat anti-rabbit (W4011, Promega).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	clone 293Ecl75 was generated in the Lingner laboratory (Lin et al., in preparation). HeLa, U2OS and 293T were from ATCC. All cell lines were tested for mycoplasma contamination.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	All raw MS data together with MaxQuant output tables are available via the ProteomXchange data repository (https://www.ebi.ac.uk/pride/archive/login) with the accession number PXD016826 (Username: reviewer18125@ebi.ac.uk; Password: ifxrqkd2).
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).	NA
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).	NA
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.	NA

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.	NA