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Looping-out Mechanism for Resolution of Replicative Stress at Telomeres

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Proposed Point-by-Point response 23 December 2016

Referee #1:

In the manuscript "Looping-out mechanism for resolution of replicative stress at telomere", Zhang et al describe an extrachromosomal telomere DNA species called t-circle-tail that consists of a circular structure with a C-rich single-stranded tail. The authors use small molecule inhibitors of DNA-PKcs and DNA ligase IV, as well as DNA-PKcs knockout, to show that formation of t-circle-tail requires NHEJ machinery. T-circle-tail formation increases in response to replication stress, leading the authors to propose a model whereby TopoII cleaves stalled telomeric replication forks to release circular DNA, and NHEJ functions to ligate the two ends together. Overall, this is an interesting study, but requires further mechanistic investigation. Specific points:
(i) There are similarities between t-circle-tail and previously reported t-complex DNA (Nabetani and Ishikawa, 2009). This should be further discussed/investigated. Is t-circle-tail a form of t-complex DNA, or is one a precursor of the other?

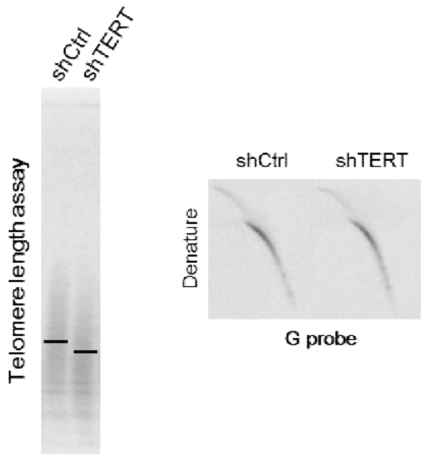
Response: T-circle-tail and T-complex are different in many aspects: 1) t-circle-tail appear as a smear on 2D gelspreading outward from the loading well Versus T-complex DNA stacked in the first dimension gel and can't migrate into second dimension gel; 2) only single-stranded C-rich DNA are present in t-circle-tail Versus both single-stranded G-rich and C-rich DNA are present in T-complex; 3) t-circle-tail is sensitive to Rec Jf (5' to 3' exonuclease) Versus T-complex is resistant. Therefore, T-complex is proposed to be a highly branched telomeric DNA with internal single-stranded G and C-rich DNA, which is structurally different from t-circle-tail consisting of double-

stranded telomeric circle and single-stranded C-rich tail. We are going to include this discussion in revised manuscript.

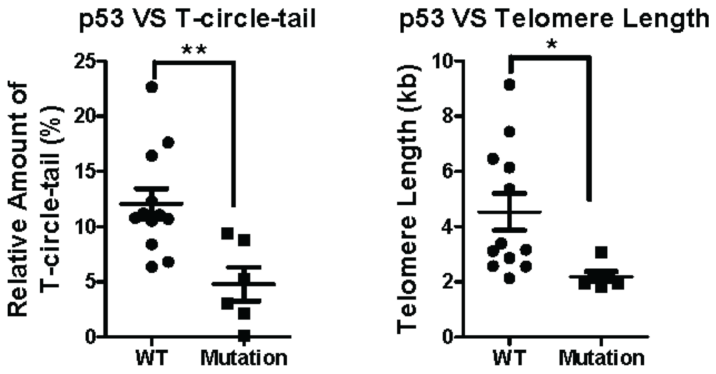
(ii) T-complex DNA is particularly prevalent in ALT cells, in which ECTR DNA is abundant. In addition, ALT cells are thought to display elevated levels of replication stress. The authors state that t-circle-tail content correlates with telomere length. Further investigation of how t-circle-tail correlates with TMM status (ie ALT or telomerase), as well as p53 status (which will determine the extent of DDR) should be included. Is there an association between t-circle-tail and telomere dysfunction (TIF analysis)? This should be explored.

Response: T-circle-tail is different in structure from T-complex, suggesting that two DNA may stem from different cellular events. T-complex is particularly prevalent in ALT positive cells, implying that it may associate with alternative lengthening of telomeres. T-circle-tail resulting from replication fork stalling at telomeres is widely present in almost all cell lines tested. We thus hypothesize that t-complex and t-circle-tail are different in function.

We are going to address reviewer's questions by providing the data we already have or by performing new experiments. 1) t-circle-tail and telomere maintenance mechanism (TMM) by telomerase: our preliminary data show that knockdown of telomerase (hTERT) has no effect on the formation of t-circle-tail (see results in the figure below), suggesting that TMM by telomerase is not involved in the formation of t-circle-tail.



2) p53 status and t-circle-tail l: We have compared the abundance of t-circle-taiil in WT-p53 and mut-p53 cell lines and found that the cells with mut-p53 have less t-circle-tail than WT-p53 cell lines (See figure below).Wee also found that mut-p53 cell lines have shorte er telomeres than WT-p53 cells (See figure bellow). The relationship between p53 and t-circle-ta ail will be further investigated by comparing the e amount of t-circle-tail in 293 cells with p53 or without p53 (siRNA knockdown of p53).



3) Telomere dysfunction foci (TIF) and t-circle-tail: We are going to induce TIF F in HeLa cells by knocking-down TRF2 to exploore how TIF affects the formation of t-circle-tail.

(iii) Were there any cell lines in which t-circle-tail was not detected? Was t there a difference between mice and human telo omeres?

Response: We found that the abundance of t-circle-tail is positively correlated t to telomere length of cells. The cells with shorter telomeres display lower level of t-circle-tail. Indeed, in human MCF-7 cells, we can hardly deetect t-circle-tail DNA (Supplementary Figure 2A).

Mouse cells contain extrremely long telomeres and the presence of an unid fentified high MW telomere-homologous signal interfered with the detection of t-circle-tail. However, telomeric signal at the expected t-circl le-tail position is also observed, indicating that t--circle-tail is also present in mice cells.

(iv) Are the 2D gels shown in Supp Fig 2A native or denatured, and which probe is used?

Response: The 2D gels in Su upp Fig 2A are hybridized with G-probe under den natured condition. We will add this information to figure legend.

(v) Many of the changes (ie t-circle-tail, telomere loss, fragile telomeres) are quite small/negligible. It is unclear how many times each of the experiments was repeated, and how reproducible the results were.

Response: Each experiment was repeated three times. The results were consistent and reproducible. Statistical analysis of data demonstrates the significant difference between the samples compared. Small change might due to low frequency of spontaneous replication fork stalling at telomeres in cells. Accordingly, t-circle-tail abundance is low and only limited number of telomere loss and/or fragile telomeres are observed.

(vi) Have the authors investigated combined inhibition of TopoII and NHEJ, is there an additive effect?

Response: This question will be addressed by following experiment: cells are treated with Topo II inhibitor or NHEJ inhibitor alone or simultaneously with two inhibitors; the amount of t-circle-tail DNA or PCNA foci at telomeres in these cells will be compared.

(vii) The increase in t-circle-tail does not appear to correlate directly with the level of induced replication stress -can the authors comment? It would be interesting to induce replication stress in the HeLa cells with different telomere lengths. This would provide a nice system to investigate t-circle-tail.

Response: As mentioned in the discussion that "many factors are recruited to telomeres to facilitate replication or to restart stalled replication forks. However, it is inevitable that replication stress due to severe DNA damage or structural obstacles will prevent at least some collapsed replication forks from being restarted", under this circumstances, "looping-out" mechanism was adopted to resolve stalled replication forks, generating t-circle-tail. In this context, the formation of t-circle-tail is not “directly” correlated with the level of induced replication stress.

We agree that the induction of replication stress in HeLa cells with different telomere lengths is a nice system to explore the formation of t-circle-tail. We are going to do this experiment. Thank the reviewer for this suggestion.

(viii) It is unclear how the authors envisage the tail in the t-circle-tail forming. Could the authors speculate further on this.

Response: The following statement will be added to revised manuscript to address this issue. "We found that the tail of t-circle-tail could be regenerated by the highly processive ϕ29 DNA polymerase, which catalyze srolling circle DNA synthesis and generates long single-stranded DNA

with a 5' free end. Although the mechanism underlying the formation of tail is remained to be elucidated, we speculated that the manner similar to rolling circle replication might occur in cells. Moreover, t-circle-tail serves as a byproduct in resolving stalled replication forks at telomeres, but what role it might play in telomere function is unknown. It has been proposed that the telomeric circular DNA may be involved in the extension of telomeres in ALT cells (Cesare & Reddel, Nat Rev Genet, 2010). In this case, the t-circle-tail with its long single-stranded C-rich strand could provide an appropriate template for synthesis and elongation of the G-rich overhang of telomeres.

As a minor point, in the methods, the authors say that they mounted their slides in DAPI -DAPI is usually used as a counterstain.

Response: It is corrected.

Referee #3:
Within this manuscript the authors propose a novel telomeric structure, the t-circle-tail. 2D electrophoresis and further characterization revealed a circular structure with a single stranded C-tail in several cell types. The abundance of the t-circle-tail was increased by replicative stress, and increasing stress and the t-circle-tail led to telomere fragility. BrdU chasing suggested that the t-circle-tail is generated by newly synthesized DNA and excised by Topisomerase 2. DNAPKcs inhibition reduced t-circle-tail frequency, suggesting a role for cNHEJ in the generation process. DNAPKcs inhibition also decreased telomere replication frequency specifically. And led to the accumulation of telomere defects. In summary, the authors provide an interesting concept by suggesting that NHEJ based resolution of stalled replication forks could lead to the excision of telomeric DNA as a t-circle-tail structure. The description of the t-circle-tail is well performed and convincing. However, the characterization of the requirement of the cNHEJ machinery and t-circle-tail excision is less convincing and needs more work.

(i) The effects of Topo 2 inhibition are weak (Figure 4), and the differences with and without the inhibitor are marginal. Can the authors demonstrate that then inhibitor actually works in these settings?

Response: The abundance of t-circle-tail is decreased by 35% in cells treated with Topo II inhibitor ICRF-187. Moreover, the treatment with merbarone, another inhibitor of Topo II, reduced the amount of t-circle-tail by 33%. Therefore, we obtained similar results using two different inhibitors.

Small difference between control and treated cells might due to the fact that the frequency of spontaneous replication fork stalling at telomeres is low. In addition, because high concentration of ICRF-187 or merbarone is toxic to cells, low dose is used in this study that inhibits a part but not all of Topo II activity.

Anyhow, we are going to perform the functional assay to validate the effectiveness of Topo II inhibitors.

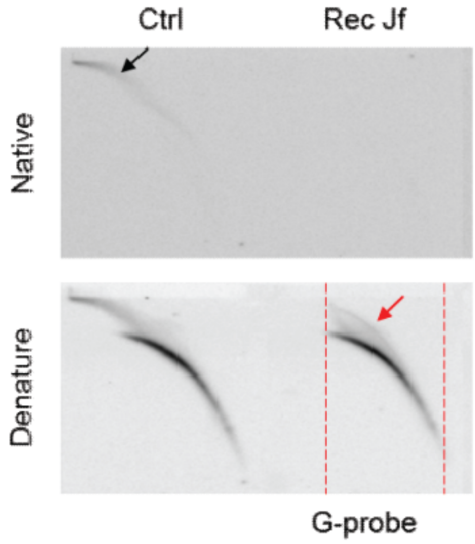
(ii) What would generate the blunt ends that can be a substrate for Ligase 4 at the telomeric breaks and the t-circle-tail?

Response: The cleavage by Topo II generates blunt end at both sides of replication fork. After stalled replication fork is excised from genome, broken telomeric DNA is provided with blunt end (see working model in Figure 7). We suppose that after release from genome, stalled replication intermediates (leading and lagging strand) should be able to complete their DNA synthesis, forming blunt ends that can be recognized and ligased by NHEJ/Ligase IV. We are going to add this to the discussion of revised manuscript.

(iii) The size distribution of the t-circle-tail is not well characterized. Considering that the fork stalling event could happen anywhere at the telomere, then cells with longer telomeres should harbor t-circle-tail structures that are larger. Can that be demonstrated?

Response: T-circle-tail consists of double-stranded circular DNA and long single-stranded C-rich tail. T-circle-tail appear as a smear on 2D gel spreading outward from the loading well. It is challenging to accurately measure the size of t-circle-tail. However, the size of circle moiety (t-

circle) of t-circle-tail is mea asurable. T-circle-tail can be converted into t-circle by digestion with RecJf. We are going to perform m the experiment to test whether the size of t-circle is correlated with telomere length. To this end, HHeLa cells with long and short telomeres will be us sed and the size of t-circle will be determined by y 2D gel analysis of purified DNA predigested wiith RecJf (see the example in figure below, t-circcle DNA is indicated by red arrow)



(iv) What are the cell cycle e effects of DNA-PKcs inhibition? Considering that thhe t-circle-tail are replication dependent, a loss o of S phase would change their frequency.

Response: The result in suppplementary Figure 8 shows that the treatment w with NU7441, an inhibitor of DNA-PKcs, has n no obvious effect on cell cycle. Therefore, the decrease of t-circle-tail is not due to cell cycle arrest t that causes the loss of S phase.

(v) What inhibits resection at the break site, which would simply be convertted into a shorter telomere? Does telomerase ex xpression alleviate the effects of Ligase 4 inhibition??

Response: Currently, we don''t know what protein inhibits end resection at the brreak site. Because there are so many proteins associating with telomeres, including telomere binding proteins, replication associated protein ns and proteins involved DNA damage repair, the e identification of protein responsible for blocki ing end resection at break site is not easy work. We e realized that this is fascinating question, but in our opinion, it falls outside of the scope of this wor rk.

Inhibition of Ligase IV leeads to decrease of t-cirlce-tail (Figure 5E) and incrreased PCNA foci at telomeres, indicating the a accumulation of telomeric replication fork stalling g (Supplementary Figure 8A and B). We are goinng to carry out the experiment to explore whether thhe overexpression of telomerase (hTERT) allevia ates the effect of ligase IV inhibition.

1st Editorial Decision 12 January 2017

Thank you for the transfer of your manuscript to EMBO reports after it was peer-reviewed for The EMBO Journal. I have read your proposed point-by-point response now, and I agree with how you plan to address the referee concerns.

I would therefore like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures, I suggest that you layout the manuscript as a normal article for which there are no length limitations. Please note that the EMBO reports reference style is numbered, this style is also in EndNote, so the current style must be corrected.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in all respective figure legends. This information is currently incomplete and must be provided in the figure legends.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

1st Revision - authors' response 03 April 2017

Referee #1:

In the manuscript "Looping-out mechanism for resolution of replicative stress at telomere", Zhang et al describe an extrachromosomal telomere DNA species called t-circle-tail that consists of a circular structure with a C-rich single-stranded tail. The authors use small molecule inhibitors of DNA-PKcs and DNA ligase IV, as well as DNA-PKcs knockout, to show that formation of t-circle-tail requires NHEJ machinery. T-circle-tail formation increases in response to replication stress, leading the authors to propose a model whereby TopoII cleaves stalled telomeric replication forks to release circular DNA, and NHEJ functions to ligate the two ends together. Overall, this is an interesting study, but requires further mechanistic investigation.
Specific points:

(i) There are similarities between t-circle-tail and previously reported t-complex DNA (Nabetani and Ishikawa, 2009). This should be further discussed/investigated. Is t-circle-tail a form of t-complex DNA, or is one a precursor of the other?

Response: Despite of potential similarities, T-circle-tail and T-complex are different in many respects: 1) t-circle-tail appears as a smear on 2D gel spreading outward from the loading well Versus T-complex DNA stacks in first dimension gel and can't migrate into second dimension gel; 2) only single-stranded C-rich DNA is present in t-circle-tail Versus both single-stranded G-rich and C-rich DNA are present in T-complex; 3) t-circle-tail is sensitive to RecJf (5' to 3' exonuclease) Versus T-complex is resistant. Therefore, T-complex is proposed to be a highly branched telomeric DNA with internal single-stranded G- and C-rich DNA, which is different from t-circle-tail that consists of double-stranded telomeric circle and single-stranded C-rich tail. This has been included in discussion section of revised manuscript (Page 14-15).

(ii) T-complex DNA is particularly prevalent in ALT cells, in which ECTR DNA is abundant. In addition, ALT cells are thought to display elevated levels of replication stress. The authors state that t-circle-tail content correlates with telomere length. Further investigation of how t-circle-tail

correlates with TMM status (ie ALT or telomerase), as well as p53 status (which will determine the extent of DDR) should be included. Is there an association between t-circle-tail and telomere dysfunction (TIF analysis)? This should be explored.

Response: T-circle-tail DNA is present in both telomerase-positive and ALT cells, suggesting that it is common mechanism for resolving replication stress at telomeres, independently of TMM. T-complex is particularly prevalent in ALT positive cells, implying that it may specifically associate with homologous recombination in ALT cells (Nabetani and Ishikawa, Mol Cell Biol, 2009).

To further investigate potential effect of telomerase on formation of t-circle-tail, we overexpressed hTERT in 293T cells and examined the change of t-circle-tail abundance. As shown in Fig EV9, we found that the amount of t-circle-tail was decreased by 30% in hTERT overexpressed cells (Fig EV9). Considering that t-circle-tail is produced by cyclization of stalled replication fork and that telomerase is recruited to broken sites of replication fork in ATM/ATR-dependent manner (Tong S, et al., Cell Rep, 2015), it is thus possible that the recruitment of telomerase interferes with NHEJ mediated t-circle-tail formation. This result was included in revised manuscript (Page 12).

We also performed the experiment to determine the effect of p53 on t-circle-tail formation. P53 was knocked down in HCT116 cells by siRNA and t-circle-tail DNA was determined by 2D gel electrophoresis. The data in Fig EV4 showed that the abundance of t-circle-tail DNA is not affected by the depletion of p53, demonstrating that p53 is not involved in the formation of t-circle-tail. This result was included in revised manuscript (Page 7-8).

To study possible correlation between telomere dysfunction induced foci (TIFs) and t-circle-tail, we induced artificial TIFs in HeLa cells by knocking out TRF2 using CRISPR/Cas9 system. New data in Supplementary Fig EV4 showed that the deficiency of TRF2 results in increased amount of TIFs in cells, but has no/limited effect on t-circle-tail formation. This result demonstrated that the generation of t-circle-tail is not caused by dysfunctional telomeres. This result was included in revised manuscript (Page 8).

(iii) Were there any cell lines in which t-circle-tail was not detected? Was there a difference between mice and human telomeres?

Response: We found that the abundance of t-circle-tail is positively correlated to telomere length of cells. The cells with shorter telomeres display fewer t-circle-tail. In human SGC cells, t-circle-tail DNA is hardly detected (0.1%) (Fig EV2A). It is thus possible that cells with even shorter telomeres may not have detectable t-circle-tail.

Mice telomeres are extremely long. T-circle-tail with much bigger size is expected in mice cells. Although the presence of an unidentified high MW telomere-homologous signal interferes with detection of t-circle-tail, telomeric signal at the expected t-circle-tail position is still observed. Furthermore, DNA-PKcs deficient mice cells display fewer t-circle-tail and more fragile telomeres, consistent with the observation in human cells.

(iv) Are the 2D gels shown in Supp Fig 2A native or denatured, and which probe is used?

Response: The 2D gels in Supp Fig 2A (Fig EV2A) are hybridized with G-probe under denatured condition. We added this information to figure legend.

(v) Many of the changes (ie t-circle-tail, telomere loss, fragile telomeres) are quite small/negligible. It is unclear how many times each of the experiments was repeated, and how reproducible the results were.

Response: The small change might be due to the fact that the frequency for resolving stalled replication fork by looping-out mechanism is low, even under stressed circumstance. Moderate replication fork stalling can be solved by conventional mechanism that induces no t-circle-tail. Only severe/irresolvable replication fork stalling needs to be resolved by loop-out mechanism.

In our experiments, each experiment was repeated three times. The results were consistent and reproducible. Statistical analysis of data demonstrates the significant difference between the samples compared. Related experimental information was extended in figure legends.

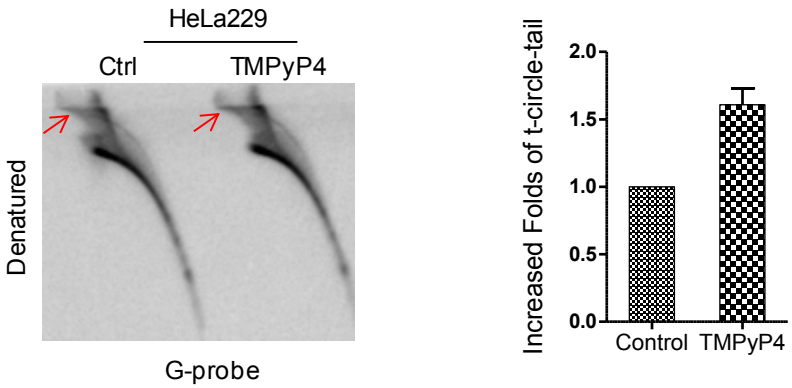
(vi) Have the authors investigated combined inhibition of TopoII and NHEJ, is there an additive effect?

Response: In Fig 6A, ICRF-187 (Topo II inhibitor) or NU7441 (DNA-PKcs inhibitor) treatment resulted in increased PCNA foci at telomeres, indicating that Topo II or NHEJ is required for successful telomere replication. To test whether additive effect exists when both Topo II and DNA-PKcs are inhibited, cells were treated with ICRF-187 and NU7441 and the increase of PCNA foci at telomeres were determined. The data in revised Fig 6A showed that combined treatment increased the percentage of cells with PCNA foci to the level approximately equal to the addition of net increases induced by ICRF-187 and NU7441 individually. This result revealed the additive effect of Topo II and NHEJ on telomere replication, implying that NHEJ machinery works in synergy with Topo II during resolution of replication fork stalling at telomeres. This result was added to revised manuscript (Page 13). We thank reviewer for this question.

(vii) The increase in t-circle-tail does not appear to correlate directly with the level of induced replication stress - can the authors comment? It would be interesting to induce replication stress in the HeLa cells with different telomere lengths. This would provide a nice system to investigate t-circle-tail.

Response: Looping-out mechanism is one of many approaches used by cells to resolve replication fork stalling at telomeres. As mentioned in the discussion that "many factors are recruited to telomeres to facilitate replication or to restart stalled replication forks. However, it is inevitable that replication stress due to severe DNA damage or structural obstacles will prevent at least some collapsed replication forks from being restarted" (Page 15), under this circumstances, "looping-out" mechanism was adopted to remove stalled replication forks, generating t-circle-tail. In this context, the formation of t-circle-tail is not “directly” correlated with the level of induced replication stress.

Using HeLa cells with different telomere lengths (HeLa, HeLa 229 and HeLa 1.2.11), we found that the greater abundance of t-circle-tail correlated with longer telomere length (Fig EV2D and E). The result suggests that replication stress (i.e. long telomeres) might promote formation of t-circle-tail. To confirm this, HeLa cells were treated with HU (Fig 2D), TMPyP4 (Fig 2F), aphidicolin (Fig EV3A), or depletion of TRF1 (Fig EV3D), all of which are able to induce replication stress at telomeres. We found that unexceptionally these treatments increased amount of t-circle-tail in cells. In addition, we also performed experiment on HeLa 229 cells using TMPyP4. TMPyP4 treatment increased the amount of t-circle-tail by 1.6 folds in HeLa 229 cells, similar to observation in HeLa cells (Fig 2F).



Taking the advantage of HeLa cells with different telomere length, we also performed experiment to determine the size of t-circle-tail, in which DNA was pre-treated with RecJf that converts t-circle-tail into telomeric circle by removing 5' single-stranded C-tail. The results showed that cells with longer telomeres harbor bigger telomeric circles (Fig EV2E), supporting the hypothesis that longer telomeres may have bigger replication fork if stalled (Page 7).

(viii) It is unclear how the authors envisage the tail in the t-circle-tail forming. Could the authors speculate further on this.

Response: The following statement has been added to discussion section of revised manuscript to address this issue (Page 17). "We found that the tail of t-circle-tail could be regenerated by the highly processiveφ29 DNA polymerase, which catalyzes rolling circle DNA synthesis and generates long single-stranded DNA with 5' free end (Fig EV1D). While the exact mechanism underlying the formation of tail remains to be demonstrated, we speculated that the manner similar to rolling circle replication might occur in cells. T-circle-tail serves as a byproduct in resolving stalled replication forks at telomeres, it might play a role during telomere lengthening. It has been proposed that the telomeric circular DNA may be involved in the extension of telomeres in ALT cells (Cesare & Reddel, Nat Rev Genet, 2010). In this case, the t-circle-tail with its long single-stranded C-rich strand could provide an appropriate template for synthesis and elongation of the G-rich overhang of telomeres".

As a minor point, in the methods, the authors say that they mounted their slides in DAPI - DAPI is usually used as a counterstain.

Response: It is corrected. Thanks.

Referee #3:

Within this manuscript the authors propose a novel telomeric structure, the t-circle-tail. 2D electrophoresis and further characterization revealed a circular structure with a single stranded C-tail in several cell types. The abundance of the t-circle-tail was increased by replicative stress, and increasing stress and the t-circle-tail led to telomere fragility. BrdU chasing suggested that the t-circle-tail is generated by newly synthesized DNA and excised by Topisomerase 2. DNAPKcs inhibition reduced t-circle-tail frequency, suggesting a role for cNHEJ in the generation process. DNAPKcs inhibition also decreased telomere replication frequency specifically. And led to the accumulation of telomere defects.

In summary, the authors provide an interesting concept by suggesting that NHEJ based resolution of stalled replication forks could lead to the excision of telomeric DNA as a t-circle-tail structure. The description of the t-circle-tail is well performed and convincing. However, the characterization of the requirement of the cNHEJ machinery and t-circle-tail excision is less convincing and needs more work.

(i) The effects of Topo 2 inhibition are weak (Figure 4), and the differences with and without the inhibitor are marginal. Can the authors demonstrate that then inhibitor actually works in these settings?

Response: ICRF-187 or merbarone has been widely used to inhibit Topo II activity *in vitro* and *in vivo* (Classen et al., PNAS, 2003) (Fortune and Osheroff, J Biol Chem, 1998). Because high dose of ICRF-187 or merbarone is toxic to cells, low dose is used in our experiments. We speculated that only a part of Topo II molecules is inhibited under this condition. The amount of t-circle-tail is decreased by 35% in cells treated with ICRF-187. Also, 33% of decrease in t-circle-tail was observed when cells were treated with merbarone. Therefore, we obtained similar results using two different inhibitors.

The small difference may also be due to the fact that the occurrence frequency for replication fork stalling that needs to be resolved by looping-out mechanism is low. Moderate replication fork stalling can be solved by conventional mechanism， only severe/irresolvable replication fork stalling requires looping-out mechanism, leading to formation of t-circle-tail.

(ii) What would generate the blunt ends that can be a substrate for Ligase 4 at the telomeric breaks and the t-circle-tail?

Response: Stalled replication fork at telomeres is excised from genome by Topo II dependent cleavage (Fig 7D). Topo II cleavage generates DNA molecules with 4 nucleotides of overhang. Further resection might be required to generate the blunt ends for Ligase IV-mediated ligation. Interestingly, it has also been reported that TDP2-mediated error-free NHEJ is an efficient and

accurate mechanism to repair Topo II-induced DSBs, suggesting that the generation of blunt ends might not be required (Gomez-Herreros, et al., PLoS Genet, 2013). Anyhow, it has been found that Ligase IV$^{-/-}$and/ or Ku70$^{-/-}$ cells are hypersensitive to Topo II poisons, highlighting the involvement of Ligase IV and Ku70 in repairing Topo II-induced DSBs (Adachi, et al., J Biol Chem, 2003). What generates the blunt ends for Ligase IV-mediated ligation is absolutely interesting question. In our opinion, it falls outside of the scope of this work.

(iii) The size distribution of the t-circle-tail is not well characterized. Considering that the fork stalling event could happen anywhere at the telomere, then cells with longer telomeres should harbor t-circle-tail structures that are larger. Can that be demonstrated?
Response: T-circle-tail consists of double-stranded circular DNA and long single-stranded C-rich tail. T-circle-tail appears as a smear on 2D gel spreading outward from the loading well. It is challenging to directly measure the size of t-circle-tail. However, the size of circle moiety (telomeric circle) of t-circle-tail can be determined by pre-treating DNA with RecJf that removes 5'-tail of t-circle-tail. We thus performed new experiment to determine the size of telomeric circle, in which HeLa cells with different telomere lengths (HeLa, HeLa 229 and HeLa 1.2.11) were used. New data showed that cells with longer telomeres harbor bigger telomeric circles (Fig EV2E), supporting the hypothesis that longer telomeres may have bigger replication fork if stalled (Page 7).

(iv) What are the cell cycle effects of DNA-PKcs inhibition? Considering that the t-circle-tail are replication dependent, a loss of S phase would change their frequency.

Response: The result in Fig EV10D and our new data in Fig EV10C showed that the treatment with NU7441, an inhibitor of DNA-PKcs, has no effect on cell cycle. Therefore, the decrease of t-circle-tail is not due to the loss of S phase (Page 13).

(v) What inhibits resection at the break site, which would simply be converted into a shorter telomere? Does telomerase expression alleviate the effects of Ligase 4 inhibition?

Response: This question is related to question ii. Currently, we don't know whether or not and how resection occurs. Since NHEJ machinery is adopted to produce t-circle-tail, we speculated that end resection is largely inhibited even if it was transiently activated. The factors responsible for suppression of end resection in NHEJ, including KU70/KU80 complex (Celli GB, et al., Nat Cell Bio, 2006), should be able to inhibit resection at the break site.

Given the fact that telomerase is recruited to stalled replication fork in ATM/ATR dependent manner (Sfeir A, et al., Cell, 2009) (Tong et al., Cell Rep, 2015), we suspected that telomerase expression might suppress the function of NHEJ. We thus performed experiment to test it and found that hTERT overexpression decreased t-circle-tail by 30% (Fig EV9), suggesting that recruitment of telomerase suppresses the formation of t-circle-tail. Consistently, when L189 was added into hTERT overexpression cells, we observed no change of t-circle-tail (Fig EV9), indicating that telomerase expression alleviates the effect of Ligase IV inhibition. The same phenotype is also observed when telomerase-overexpressed cells were treated with NU7441 (Fig EV9). We thank this reviewer for this question. The relative data were added to Fig EV9 (Page 12).

2nd Editorial Decision 20 April 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from 2 of the original referees, which are pasted below. As the referees are not in agreement, I also contacted a member of our advisory board, who agrees with referee 2 that the findings are interesting and who does not raise any concerns. We can therefore in principle accept your manuscript for publication.

A few more changes are required: The main figures need to be uploaded as individual files and need to be changed to portrait format. Each figure should fit on a printed page.

The 10 supplemental figures can be published in an Appendix file. Alternatively, 5 supplementary figures can be changed into EV (expanded view) figures that are embedded in the main manuscript text and expand when clicked online. EV figures need to be uploaded as separate files and the EV

legends added to the end of the main manuscript text. The remaining figures will need to be moved to the Appendix file. You can find more information in our guide to authors online.

The zoomed image in figure 7A top is flipped, and the zoomed boxes in the bottom image are misplaced in the original image. In figure 6D some images in the boxes are flipped too.

Figures EV6, EV8 and 5C seem to have black patches on the image, please explain.

In figure EV2C if bands were cut out from this gel, please indicate by leaving some white space between the gel pieces.

Please check very carefully that all relevant figure panels include the required information on the statistic analyses and that all microscopy images have scale bars.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

Overall, the authors have put great effort into addressing the specific question raised by both reviewers, and have done a really good job. However, I remain unconvinced by some of the documented changes in t-circle-tail (for instance in EV9 (B) and (D)), the mechanistic origin of t-circle-tail and the relevance of this form of telomeric DNA.

Referee #2:

I am satisfied with the revision and support publication bin the current form.

2nd Revision - authors' response 29 April 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from 2 of the original referees, which are pasted below. As the referees are not in agreement, I also contacted a member of our advisory board, who agrees with referee 2 that the findings are interesting and who does not raise any concerns. We can therefore in principle accept your manuscript for publication.

A few more changes are required:
The main figures need to be uploaded as individual files and need to be changed to portrait format. Each figure should fit on a printed page.
Response: Figures are changed to portrait format and every figure was uploaded as an individual file.

The 10 supplemental figures can be published in an Appendix file. Alternatively, 5 supplementary figures can be changed into EV (expanded view) figures that are embedded in the main manuscript text and expand when clicked online. EV figures need to be uploaded as separate files and the EV legends added to the end of the main manuscript text. The remaining figures will need to be moved to the Appendix file. You can find more information in our guide to authors online.
Response: Please publish all 10 supplementary figures as a single appendix file.

The zoomed image in figure 7A top is flipped, and the zoomed boxes in the bottom image are misplaced in the original image. In figure 6D some images in the boxes are flipped too.
Response: All are corrected.

Figures EV6, EV8 and 5C seem to have black patches on the image, please explain.
Response: The black patches were caused by low resolution of images. When these images were

converted into PDF, the black squares appeared that cover some dots in image. We had replaced images involved with high resolution pictures.

In figure EV2C if bands were cut out from this gel, please indicate by leaving some white space between the gel pieces.
Response: It is corrected.

Please check very carefully that all relevant figure panels include the required information on the statistic analyses and that all microscopy images have scale bars.
Response: We double-checked and confirmed that all the information on statistic analysis were included in figure legends and that all microscopy images include scale bars.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.
Response: The synopsis image, summary and highlights were uploaded along with the revised manuscript.

3rd Editorial Decision 08 May 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our 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: Yong Zhao
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43866-T

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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 justified
- 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	In each experiment, a sample size was chosen to determine tatistical significance by students' unpaired t-test.
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.	NA
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.	Students' unpaired t-test
Is there an estimate of variation within each group of data?	Mean± SEM (n=3) was used for statistical analysis.
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

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).	Catalog numbers for all antibodies used are listed in the methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa and HeLa229 and majority of cell lines in Fig EV2 were purchased from Chinese Academy of Sciences of Type Culture Collection. All cell lines were tested for free of microplasma before experiments. HTC75 was from Zhou Songyang's lab, HeLa1.2.11 was from Carolyn M Price's lab.

* 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 accession codes for deposited data. See author guidelines, under '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	NA
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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

23. 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