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Molecular Steps of G-overhang Generation at Human Telomeres and Its Function in Chromosome End Protection

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 15 February 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, these referees considers your findings on G-overhang generation at human telomeres interesting and potentially important. On the other hand, they raise a number of substantive criticism, which appear to be well-taken and to currently preclude publication. Taking all the opinions and recommendations at hand into account, I feel we should be able to consider a revised version for publication if you should be able to satisfactorily address these main experimental concerns, especially the issues raised by referee 1 and the main point of referee 2 regarding a more direct addressing of the proposed leading strand processing. In this respect, please however note that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be essential to thoroughly answer to these experimental criticisms, as well as to adequately respond to the various other specific points raised by the reviewers, at this stage of the process. Finally, given the partial overlap with the mentioned paper by Zhao et al in Cell, it would also be important to swiftly proceed with the revision of the present study, and I am therefore hoping you will be able to resubmit a revised version as early as possible.

When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Summary:

In this manuscript, Chai et. al. analyze the telomeric G-rich overhang in human cells throughout the cell cycle and investigate the mechanism of subsequent fill-in synthesis of the C-rich strand in late S phase. Their experiments confirm previous reports that the single stranded G-rich overhang lengthens in late S/G2, independently of telomerase, and shortens after completion of C-strand fill-in. They find, surprisingly, that the last nucleotide of the C-rich strand is tightly regulated throughout S phase. They present evidence that aphidicolin treatment and CDK inhibition lead to persistence of the G-rich overhang as well as signs of DNA damage. Finally, they determine the contribution of Stn1 to overhang dynamics.

Comments:

The authors' analysis of the G-rich overhang throughout S phase confirm previously published data on overhang length changes during the cell cycle. Their analysis of the last nucleotide of the C-strand in synchronized cells progressing through S phase suggests that processing of the C-rich strand is tightly regulated. This finding is surprising and interesting, though it could be argued that the replication of the Xp/Yp telomere may not be synchronized across cells even if bulk replication is synchronized. Nonetheless, the results of STELA at late S timepoints, when most of the Xp/Yp telomeres have completed replication but prior to C-strand fill-in, support tight coupling of C strand processing to replication.

The major critique of this manuscript comes in the latter half, with the experiments aimed at elucidating the mechanism of C-strand fill-in. Though these are important investigations, these experiments contain major flaws that preclude conclusions without additional support using alternative experimental methods. I cannot recommend this manuscript for publication until these concerns are addressed.

The experiments (in Fig. 4) to test the involvement of lagging strand machinery in C-strand fill-in use aphidicolin to inhibit DNA polymerases alpha and delta. However, aphidicolin treatment at 6h after release from G1/S induces a G2/M arrest . The persistence of a long overhang could be due to the cell cycle arrest and not specifically the inhibition of DNA lagging strand synthesis polymerases. The authors' conclusion that the lagging strand machinery is required for C-strand fill-in requires support from additional experiments that would more specifically inhibit the lagging strand machinery during late S/G2 without inducing a cell cycle arrest, and determining whether long overhangs still persist even after completion of mitosis. Similarly, inhibiting CDK using purvanalol (Fig. 5) also appeared to arrest cells in G2/M, so it is unclear whether the effect of CDK inhibition is also due to the cell cycle arrest.

The experiments aimed at determining negative consequences of persistent excess single-stranded signal due to failure of C-strand fill-in are also not interpretable (Fig. 6). As the authors themselves acknowledged, aphidicolin itself induces an ATR-dependent DNA damage signal at telomeres. Thus, the increase in gamma-H2AX foci after aphidicolin treatment cannot be solely attributed to the persistent overhang. The number of H2AX foci that colocalize with TRF2 in response to purvalanol treatment is extremely low (only ~10% have >3 co-localized foci - would this percentage be less with a cutoff of 5 or 10, commonly used in the literature?). It is unclear whether this increase is statistically or physiologically significant.

Finally, the experiments knocking down Stn1 are uninterpretable since the cells are apoptotic at the time of the assays. There is no information on whether the induced gamma-H2AX foci localize with telomeres.

Referee #2 (Remarks to the Author):

This manuscript examines the process of G-overhang formation during telomere replication. The authors use assays for G-overhang length and the identity of the terminal nucleotide on the resected C-strand to analyze G-overhang structure as cells progress through S-phase into G2/M. The work builds on earlier work by Dr Chai and a recent publication from her postdoctoral mentor's lab (Zhao et al, Cell 138, 2009). The current manuscript describes a number of novel findings that shed light on the steps in overhang generation in mammalian cells. The major findings are (i) the increase in overhang length observed in S-phase can occur in the absence of telomerase and hence can solely reflect DNA processing. (ii) The terminal sequence of the C-strand is constant throughout S-phase indicating that C-strand processing is tightly regulated. (iii) Pol alpha primase is needed for the shortening of the G-overhangs that occurs in S/G2. Moreover, this C-strand fill-in reaction requires the action of cyclin-dependent kinase 1. (iv) Inhibition of C-strand fill-in results in residual long G-overhangs which elicit a DNA damage response.
Taken together these findings provide an important confirmation that processing of telomeric DNA occurs in conjunction with DNA replication while C-strand fill-in is delayed until late S/G2 . The work also provides novel insight into the regulation of G-overhang formation by demonstrating that C-strand processing is not regulated by CDK whereas C-strand fill-in by Pol alpha does depend on CDK activity. The early CDK-independent processing is quite different from what has been observed in budding yeast where telomere processing, telomerase action and C-strand fill-in all occur in late S and are dependent on CDk activity.
Overall this is a nice, carefully performed piece of work. I have only one substantial criticism. This is that the authors do not show directly that the leading strand telomeres are processed. Bunt-ended leading strand telomeres would not show up in the DSN G-overhang or STELLA assays so in theory they could just be looking at lagging strand telomeres.
In this respect, the manuscript leans heavily on the Zhao 2009 Cell paper from the Wright/Shay lab. The Zhao paper showed telomerase extension of leading strand telomeres, which in turn indicates that some processing must have taken place. However, Zhao et al. did not address how many nucleotides were removed. Since telomerase only needs a very short substrate (6-8 nt), it is possible that the leading strand telomeres had very short overhangs. In which case the DSN assays used in the current manuscript might only be able to detect lagging strand telomeres. Thus, the current study would be more robust if the authors would separate leading from lagging strand telomeres (technology pioneered by Dr Chai) prior to looking at overhang length and the terminal sequence on the C-strand.
Other minor comments
1. The authors should show the cell synchronization data for Fig. 3 in supplemental data. It would be helpful to include in Fig. 3 a diagram illustrating the STELLA protocol.
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3. Fig. 4. Mention more clearly (e.g. in the figure legend) that the HeLa cells were synchronized in G1/S and then released.
4. Fig 7D. It is not at all clear that the cells in the image are undergoing apoptosis. The authors need to either show a higher magnification or (preferably) more specific staining such as tunnel or annexin.
5. Page 18, last line of the first paragraph. The meaning of this is unclear.
6. Page 18 paragraph 2. Presumably the authors mean that the G-overhang must be bound and protected by proteins other than RPA because RPA binding is the first step in ATR recruitment and the ensuing DNA damage response.

Referee #3 (Remarks to the Author):

G-overhangs play pivotal roles in chromosome end protection and in promoting telomerase action to maintain telomeric DNA. In this manuscript, Chai and colleagues examine the regulation of the G-

overhang at human telomeres. They demonstrate that the length of G-overhangs is dynamically regulated during the cell cycle, while the C-strand terminal nucleotide is precisely defined. Further they identify Cdk1 as an important regulator of C-strand fill in. Overall, this is a carefully conducted study with potentially important ramifications for the telomere field.

Specific Comments:
1. The authors argue that extended G-overhangs are due to C-strand resection. However, this conclusion is not based on direct experimental support, but rather on exclusion of other possibilities. The conclusion needs to be softened.

2. Zhao et al (2009) showed evidence of telomerase involvement in G-overhang extension. In contrast, the authors show that extension of G-overhang is independent of telomerase in human cells. Some explanation for this discrepancy is required.

3. In Fig 5A&B, the delayed reduction of G-overhang length at 12hr in the presence of purvalanol is correlated with a G2 cell cycle arrest in 5C. Can the authors rule out the possibility that there is a defect in C-strand fill-in, or G2 arrest or some other mechanism that indirectly blocks C-strand processing?

4. More information is needed concerning the Pot1 and Stn1 RNAi experiments. How much mRNA/protein depletion was achieved?

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6. For Stn1 RNAi, a figure of gamma-H2AX staining needs to be included in Fig 7 which addresses whether the staining localizes with telomeres.

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1. Results with Stn1 mutants should be mentioned in the abstract.

2. On pages 4 and 16, single-cell eukaryote might be more accurate than "lower eukaryote".

3. The last sentence in the introduction is unnecessary and could be deleted..

4. On page 19, the correct statement is "Ctc1/Stn1 stimulates pol activity in vitro". In the Casteel et al manuscript, the two proteins were assayed together for activity.

1st Revision - authors' response 14 May 2010

Response to reviewer’s comments:

We thank the reviewers for providing many insightful comments that have helped us improve the original submission. We have added new data to this revised manuscript that greatly strengthens the significance of this research and drastically reduces the overlap with published paper. To help reviewers discriminate between text from the original submission and revised version, we have added side-bars to the left margin to indicate where the manuscript has been significantly changed.

Referee #1:
Summary:

In this manuscript, Chai et. al. analyze the telomeric G-rich overhang in human cells throughout the cell cycle and investigate the mechanism of subsequent fill-in synthesis of the C-rich strand in late S phase. Their experiments confirm previous reports that the single stranded G-rich overhang lengthens in late S/G2, independently of telomerase, and shortens after completion of C-strand fill-

in. They find, surprisingly, that the last nucleotide of the C-rich strand is tightly regulated throughout S phase. They present evidence that aphidicolin treatment and CDK inhibition lead to persistence of the G-rich overhang as well as signs of DNA damage. Finally, they determine the contribution of Stn1 to overhang dynamics.

Comments:

The authors' analysis of the G-rich overhang throughout S phase confirm previously published data on overhang length changes during the cell cycle. Their analysis of the last nucleotide of the C-strand in synchronized cells progressing through S phase suggests that processing of the C-rich strand is tightly regulated. This finding is surprising and interesting, though it could be argued that the replication of the Xp/Yp telomere may not be synchronized across cells even if bulk replication is synchronized. Nonetheless, the results of STELA at late S timepoints, when most of the Xp/Yp telomeres have completed replication but prior to C-strand fill-in, support tight coupling of C strand processing to replication.

The major critique of this manuscript comes in the latter half, with the experiments aimed at elucidating the mechanism of C-strand fill-in. Though these are important investigations, these experiments contain major flaws that preclude conclusions without additional support using alternative experimental methods. I cannot recommend this manuscript for publication until these concerns are addressed.

1. The experiments (in Fig. 4) to test the involvement of lagging strand machinery in C-strand fill-in use aphidicolin to inhibit DNA polymerases alpha and delta. However, aphidicolin treatment at 6h after release from G1/S induces a G2/M arrest. The persistence of a long overhang could be due to the cell cycle arrest and not specifically the inhibition of DNA lagging strand synthesis polymerases. The authors' conclusion that the lagging strand machinery is required for C-strand fill-in requires support from additional experiments that would more specifically inhibit the lagging strand machinery during late S/G2 without inducing a cell cycle arrest, and determining whether long overhangs still persist even after completion of mitosis. Similarly, inhibiting CDK using purvanalol (Fig. 5) also appeared to arrest cells in G2/M, so it is unclear whether the effect of CDK inhibition is also due to the cell cycle arrest.

It seems to us that the reviewer's concern is whether failure of C-strand fill-in was due to the inability of cells to go through G2/M because both aphidicolin and purvalanol treatments arrested cells at G2/M. We thank this reviewer for the comments. To address this concern, we have treated cells with two different small molecules, nocodazole, an inhibitor of microtubule, and etoposide, an inhibitor of topoisomerase II, at late S phase. As shown in Fig. S2, treatment with either inhibitor arrested cells at G2/M, and G-overhangs were not elongated after the treatment, suggesting that the effect of pol inhibition or CDK1 inhibition on G-overhang was not due to the cycle arrest. In addition, when cells were treated with caffeine concurrently with aphidicolin, about half of cells bypassed G2/M arrest. (We had tried various conditions of caffeine and aphidicolin and also tested adding caffeine at different times during the cell cycle to try to make cells bypass the G2/M arrest. However, our best effort is to make about half of cells bypass the arrest.) If pol was not required for C-strand fill-in, it was expected that overhangs would become shorter as ~50% of cells went through G2/M to the next G1. However, Fig. S2 shows that lengthened G-overhangs still persisted. Therefore, we conclude that the aphidicolin-induced G-overhang lengthening was not due the inability of cells to go through G2/M. We have added the results in the manuscript on p12-13 and Fig. S2.

2.The experiments aimed at determining negative consequences of persistent excess single-stranded signal due to failure of C-strand fill-in are also not interpretable (Fig. 6). As the authors themselves acknowledged, aphidicolin itself induces an ATR-dependent DNA damage signal at telomeres. Thus, the increase in gamma-H2AX foci after aphidicolin treatment cannot be solely attributed to the persistent overhang.

We totally agree with the reviewer that the increase in -H2AX foci after aphidicolin treatment cannot be solely attributed to the lengthened overhang, as we stated in the original manuscript. We have eliminated Fig. 6A (Fig. 7 in revised manuscript) and corresponding text. This is the exact reason that we used CDK1 inhibition to analyze DNA damage response. The reviewer is correct

that our interpretation in the original manuscript may be overstated. To be more precise, we have changed the subtitle from the original "Excessive ss G-overhangs lead to DNA damage response at telomeres" to the revised "CDK1 inhibition leads to DNA damage response at telomeres" on p14.

3. The number of H2AX foci that colocalize with TRF2 in response to purvalanol treatment is extremely low (only ~10% have >3 co-localized foci - would this percentage be less with a cutoff of 5 or 10, commonly used in the literature?). It is unclear whether this increase is statistically or physiologically significant.

We thank the reviewer for pointing this out. We have repeated the experiment two more times. Statistical analysis is now included in Fig. 7C (original Fig 6B). Telomeric DNA damage response with >5 TRF2/gamma-H2AX co-localization is clearly present in purvalanol treated cells. We have noticed that most literatures use co-localization of 4 or 5 as a cutoff (d'Adda di Fagagna et al, 2003; Denchi & de Lange, 2007; Sfeir et al, 2009; Takai et al, 2003), while some literatures used bigger numbers as a cutoff (Konishi & de Lange, 2008). To be precise, we have provided information on the percent of cells containing 5-7 co-localizations and 8 co-localizations in Fig. 7C. We hope the reviewer find the information satisfying.

4. Finally, the experiments knocking down Stn1 are uninterpretable since the cells are apoptotic at the time of the assays. There is no information on whether the induced gamma-H2AX foci localize with telomeres.

We have performed co-localization of gamma-H2AX with TRF2 and included data in Figs. 8D and S3.

Referee #2

This manuscript examines the process of G-overhang formation during telomere replication. The authors use assays for G-overhang length and the identity of the terminal nucleotide on the resected C-strand to analyze G-overhang structure as cells progress through S-phase into G2/M. The work builds on earlier work by Dr Chai and a recent publication from her postdoctoral mentor's lab (Zhao et al, Cell 138, 2009). The current manuscript describes a number of novel findings that shed light on the steps in overhang generation in mammalian cells. The major findings are (i) the increase in overhang length observed in S-phase can occur in the absence of telomerase and hence can solely reflect DNA processing. (ii) The terminal sequence of the C-strand is constant throughout S-phase indicating that C-strand processing is tightly regulated. (iii) Pol alpha primase is needed for the shortening of the G-overhangs that occurs in S/G2. Moreover, this C-strand fill-in reaction requires the action of cyclin-dependent kinase 1. (iv) Inhibition of C-strand fill-in results in residual long G-overhangs which elicit a DNA damage response.

Taken together these findings provide an important confirmation that processing of telomeric DNA occurs in conjunction with DNA replication while C-strand fill-in is delayed until late S/G2. The work also provides novel insight into the regulation of G-overhang formation by demonstrating that C-strand processing is not regulated by CDK whereas C-strand fill-in by Pol alpha does depend on CDK activity. The early CDK-independent processing is quite different from what has been observed in budding yeast where telomere processing, telomerase action and C-strand fill-in all occur in late S and are dependent on CDk activity.

Overall this is a nice, carefully performed piece of work. I have only one substantial criticism. This is that the authors do not show directly that the leading strand telomeres are processed. Blunt-ended leading strand telomeres would not show up in the DSN G-overhang or STELLA assays so in theory they could just be looking at lagging strand telomeres.

In this respect, the manuscript leans heavily on the Zhao 2009 Cell paper from the Wright/Shay lab. The Zhao paper showed telomerase extension of leading strand telomeres, which in turn indicates that some processing must have taken place. However, Zhao et al. did not address how many nucleotides were removed. Since telomerase only needs a very short substrate (6-8 nt), it is possible that the leading strand telomeres had very short overhangs. In which case the DSN assays used in the current manuscript might only be able to detect lagging strand telomeres. Thus, the current

study would be more robust if the authors would separate leading from lagging strand telomeres (technology pioneered by Dr Chai) prior to looking at overhang length and the terminal sequence on the C-strand.

We thank this reviewer for providing the excellent insights. As suggested, we have separated leading and lagging daughter telomeres using CsCl gradient ultracentrifugation and determined the cell cycle-regulated G-overhang dynamics at the two daughter telomeres. The major finding is that leading and lagging telomeres display different G-overhang dynamics during the cell cycle. While lagging overhangs are elongated during S phase and then shortened in late S/G2 due to C-strand fill-in, leading overhangs remains short throughout S phase and the elongation of leading overhangs is delayed until G2/M phase. The elongated leading overhangs do not appear to experience further shortening, thus no C-strand fill-in seems to take place at leading telomeres. Data are included in Fig. 3 as a new figure and extensive description of results and discussion are provided on p8-9 and p18-19. At present the underlying mechanism for this differential G-overhang generation at leading and lagging telomeres is unknown. Since separation of leading and lagging telomeres is not a trivial effort, we think providing more data for understanding the mechanism for this difference is beyond the scope of this manuscript. We sincerely hope the reviewer will consider the amount of work we have included in the revised manuscript within three months.

We did attempt to do STELA with purified leading and lagging telomeres. Unfortunately, we found that the triple enzyme digestion of genomic DNA prior to CsCl density ultracentrifugation removed the annealing site for XpYpE2 primer. Therefore, the ligated products could not be amplified by PCR. However, we think that performing STELA on separated leading and lagging telomeres is not imperative for the current manuscript. Our STELA results from unseparated telomeres are sufficient for representing the specificity of last C from all the measurable telomeres. Since C-telorettes only anneal to G-overhangs of 6nt length, STELA does not measure three populations of telomere ends: unprocessed blunt-ended leading telomeres, processed leading telomeres with < 6nt overhangs, and unprocessed RNA primer-containing lagging telomeres. Therefore, even if we use separated leading and lagging XpYp telomeres for STELA, telomeres containing the above ends still cannot anneal to C-telorretes and therefore will not be measured by STELA anyway.

Because current G-overhang measuring techniques are unable to determine the size of G-overhangs smaller than 6 nt, we do not know the population of telomeres containing extremely short G-overhangs. Nevertheless, regardless of the limitation of STELA, the last Cs measured here still reflects the specificity at all the measurable telomeres.

It seems to us that the reviewer is interested in knowing the size of leading overhangs. We have compared the overhang abundance at leading/lagging telomeres (Fig. 3D) and the calculated mean length of lagging overhangs (data not shown), we calculated that the average overhang length at leading telomeres during S phase is about 30 nt, which becomes longer in G2/M. We decide not to include this information in the revised manuscript because we were unable to determine the absolute lengths of G-overhangs at leading telomeres or the distribution of extremely short versus longer leading overhangs.

Other minor comments

1. The authors should show the cell synchronization data for Fig. 3 in supplemental data. It would be helpful to include in Fig. 3 a diagram illustrating the STELLA protocol.

We used the same DNA from Fig. 1 for STELA analysis in Fig. 3. FACS is provided in Fig. 1A. We have added a diagram of STELA in Fig. 4 (original Fig. 3) as suggested.

2. Page 9. Zhao et all (Cell 2009) showed that the XpYp telomere was replicated 2-4 hours after release from G1/S. This means that the XpYp telomere will almost certainly have been replicated during the time frame examined in the current manuscript.

We thank the reviewer for pointing this out. We have modified the manuscript and added the following on p11: "Since XpYp telomere replicates between 2 to 4 hr after release into S phase (Zhao et al, 2009), the last C at 1.5 hr after release mainly arose from unreplicated XpYp telomere, last C measured at 3 hr resulted from a mixture of newly replicated and unreplicated XpYp, while last C measured at 4.5 hr and afterward represented fully replicated telomere."

3. Fig. 4. Mention more clearly (e.g. in the figure legend) that the HeLa cells were synchronized in G1/S and then released.

We have changed this in Figure legend on p28 (current Fig. 5).

4. Fig 7D. It is not at all clear that the cells in the image are undergoing apoptosis. The authors need to either show a higher magnification or (preferably) more specific staining such as tunnel or annexin.

We performed annexin V-FITC staining as suggested and the data is now included in Fig. 8E.

5. Page 18, last line of the first paragraph. The meaning of this is unclear.

We apologize for the unclearness. We have modified the paragraph as the following on p19: "Alternatively, C-strand fill-in may be the last step of telomere DNA replication. Until DNA polymerases finish C-strand fill-in and depart from telomeres, cells might consider DNA replication was incomplete and the checkpoint pathways would signal a cell cycle arrest."

6. Page 18 paragraph 2. Presumably the authors mean that the G-overhang must be bound and protected by proteins other than RPA because RPA binding is the first step in ATR recruitment and the ensuing DNA damage response.

The reviewer is absolutely correct. The sentence has been changed to the following: "The S/G2 phase-specific excessive ss G-overhangs ought to be protected by proteins other than RPA, as RPA binding may trigger ATR-dependent DNA damage response."

Referee #3

G-overhangs play pivotal roles in chromosome end protection and in promoting telomerase action to maintain telomeric DNA. In this manuscript, Chai and colleagues examine the regulation of the G-overhang at human telomeres. They demonstrate that the length of G-overhangs is dynamically regulated during the cell cycle, while the C-strand terminal nucleotide is precisely defined. Further they identify Cdk1 as an important regulator of C-strand fill in. Overall, this is a carefully conducted study with potentially important ramifications for the telomere field.

Specific Comments:
1. The authors argue that extended G-overhangs are due to C-strand resection. However, this conclusion is not based on direct experimental support, but rather on exclusion of other possibilities. The conclusion needs to be softened.

The reviewer is correct. We have modified our conclusion throughout the manuscript as well as our model.

2. Zhao et al (2009) showed evidence of telomerase involvement in G-overhang extension. In contrast, the authors show that extension of G-overhang is independent of telomerase in human cells. Some explanation for this discrepancy is required.

We have provided the following explanation in the revised manuscript on p19: "We have observed that the telomerase negative cell line IMR90 exhibits transient G-overhang lengthening in S phase similar to telomerase positive cells (Fig. 1), suggesting that telomerase action is not required for generating elongated lagging overhangs. The telomerase-independent G-overhang lengthening may be caused by two reasons. During replication, if the final RNA primer is not placed at the very end of chromosome DNA, a long G-overhang may be generated. Alternatively, extensive resection at C-strand of lagging telomeres following replication may also create excessive ss G-overhangs."

3. In Fig 5A&B, the delayed reduction of G-overhang length at 12hr in the presence of purvalanol is correlated with a G2 cell cycle arrest in 5C. Can the authors rule out the possibility that there is a

defect in C-strand fill-in, or G2 arrest or some other mechanism that indirectly blocks C-strand processing?

This is the same as reviewer 1's comment #1. Please see detailed explanation in the response to reviewer 1. The data is now shown in Fig. S2.

4. More information is needed concerning the Pot1 and Stn1 RNAi experiments. How much mRNA/protein depletion was achieved?

We have added the requested information below the western blot images in Fig. 4 and Fig. 8.

5. In Fig 6 and in text on page 14: what proportion of gamma-H2AX signal co-localized with TRF2 (or telomeres)?

This is the same as reviewer 1's comment #3. Please see detailed explanation in the response to reviewer 1. The data is now shown in Fig. 7C.

6. For Stn1 RNAi, a figure of gamma-H2AX staining needs to be included in Fig 7 which addresses whether the staining localizes with telomeres.

We have done so as suggested and the data is included in Figs. 8D and S3. Text description has been added in p15.

Minor Comments:
1. Results with Stn1 mutants should be mentioned in the abstract.

We have done so as suggested.

2. On pages 4 and 16, single-cell eukaryote might be more accurate than "lower eukaryote".

In the majority of literature, the expression of "lower eukaryote" is used. Therefore, we decide to keep this convention.

3. The last sentence in the introduction is unnecessary and could be deleted.

It's deleted.

4. On page 19, the correct statement is "Ctc1/Stn1 stimulates polα activity in vitro". In the Casteel et al manuscript, the two proteins were assayed together for activity.

We thank the reviewer for pointing this out. We have changed the manuscript to reflect accuracy.

References:
d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP (2003) A DNA damage checkpoint response in telomere-initiated senescence. Nature 426: 194-198

Denchi EL, de Lange T (2007) Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature 448: 1068-1071

Konishi A, de Lange T (2008) Cell cycle control of telomere protection and NHEJ revealed by a ts mutation in the DNA-binding domain of TRF2. Genes Dev 22: 1221-1230

Sfeir A, Kosiyatrakul ST, Hockemeyer D, MacRae SL, Karlseder J, Schildkraut CL, de Lange T (2009) Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. Cell 138: 90-103

Takai H, Smogorzewska A, de Lange T (2003) DNA damage foci at dysfunctional telomeres. Curr Biol 13: 1549-1556

Zhao Y, Sfeir AJ, Zou Y, Buseman CM, Chow TT, Shay JW, Wright WE (2009) Telomere extension occurs at most chromosome ends and is uncoupled from fill-in in human cancer cells. Cell 138: 463-475

2nd Editorial Decision 07 June 2010

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the original reviewer 2, and I am happy to inform you that in light of their comments (please see below) we feel that the manuscript is now suitable for in The EMBO Journal in principle. As you will see, there remain however a few specific issues that would still need to be addressed. While I do not feel that this requires further experimentation, I would like to ask you to carefully address these - largely editorial - issues, including toning down of some of the conclusions and providing a more in-depth discussions of your results in light of divergent findings from other reports. In addition, the manuscript would also still benefit from additional in-depth editing ideally by a native English-speaking scientists.

I am therefore returning the study to you once more, kindly inviting you to incorporate these additional changes into the manuscript text and to return the final version to us at your earliest convenience. After that, we should be able to swiftly proceed with its formal acceptance and production.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

This manuscript has undergone substantial revision since the last submission and a number of new experiments have been included. In some ways these revisions have strengthened the manuscript, but other revisions have raised further questions.

The important contributions remain: (i) the work provides an important confirmation that processing of telomeric DNA to create the G-strand overhang occurs in conjunction with DNA replication while C-strand fill-in occurs later on in late S/G2. (ii) It provides new insight into the regulation of G-overhang formation by showing that C-strand fill-in but not C-strand processing is regulated by DNA pol alpha.

The new experiments showing that cell cycle arrest in late S or G2 does not prevent C-strand fill-in answer a key criticism by reviewers 1 and 3 who suggested that the lack of fill-in might simply be caused by the cell cycle arrest rather than inhibition of DNA-pol alpha or Cdk.

The experiment where the authors separate leading from lagging strand telomeres and analyze overhang length by in gel hybridization answers the criticism from reviewer 2 that the authors did not directly show that leading strand telomeres are processed. The authors now demonstrate that overhangs are indeed present on the leading strand telomeres so they must have been processed. They do not answer the question as to whether the terminal sequence of the leading strand telomeres is maintained after processing. This is a pity, but understandable given the technical problems. However, what is disturbing is that the results differ from those obtained from a similar (but not identical) experiment published in the Zhao 2009 Cell paper. Zhao et al report a net increase in overhang length at leading strand telomeres during S-phase while the authors of the current

manuscript report a decrease in length. While the timing of the analysis done by Zhao et al. was somewhat different, it is very difficult to reconcile the results from the two labs. Unfortunately, the authors of the current manuscript simply ignore this discrepancy. Clearly it was an unexpected result and one which they do not appear to have come to grips with as they mention telomerase extension of leading strand telomeres in the discussion on page 16 (line 9) but yet say elsewhere that their results indicate that this does not occur.

The type of analysis described in this manuscript and by Zhao et al. is technically very difficult and prone to artifacts. Thus, there is currently no way to tell which lab is correct. For this reason, I would recommend leaving the leading versus lagging strand telomere analysis as part of the paper. However, I think the authors should address the discrepancy with the Zhao results and tone down their conclusions.

Another concern revolves around the author's conclusion that the DNA damage response caused by the purvalanol treatment results from the accumulation of single-stranded G-strand DNA. The actual increase in G-overhang is fairly modest (<50%) so there may well be enough Pot1 (or CST) in the cell to coat the extra overhang and exclude RPA. An alternative reason for the damage response might be that the purvalanol treatment also inhibits processes needed to resolve other replication defects. These could include replication fork stalling in the telomeric tract which would then result in telomeric gamma H2Ax foci.

Other comments

Page 8, line 18. The extended G-overhangs could be due to telomerase extension in addition to C-strand resection as the experiment was done in HeLa cells. To draw a conclusion that the extended G-overhangs in S-phase were due to extensive resection, the experiment would have to be repeated in telomerase negative cells.

Page 17, line 5. Remove "first" as other work has also provided insights into G-overhang generation at leading and lagging daughter telomeres.

Legend to Figure 2 B. Remove "comparing to DMSO" (page 28, line 4) as this is confusing. The FACS profile is shown beside (not below) the G-overhang analysis.

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2nd Revision - authors' response 17 June 2010

We thank the reviewer for providing insightful comments that have helped us to improve the original submission. We have revised the text in the manuscript to address the reviewer's comments. To help the reviewer discriminate between text from the original submission and revised version, we have added side-bars to the left margin to indicate where the manuscript has been significantly changed.

Referee #2 (Remarks to the Author):

This manuscript has undergone substantial revision since the last submission and a number of new experiments have been included. In some ways these revisions have strengthened the manuscript, but other revisions have raised further questions.
The important contributions remain: (i) the work provides an important confirmation that processing of telomeric DNA to create the G-strand overhang occurs in conjunction with DNA replication while C-strand fill-in occurs later on in late S/G2. (ii) It provides new insight into the

regulation of G-overhang formation by showing that C-strand fill-in but not C-strand processing is regulated by DNA pol alpha.
The new experiments showing that cell cycle arrest in late S or G2 does not prevent C-strand fill-in answer a key criticism by reviewers 1 and 3 who suggested that the lack of fill-in might simply be caused by the cell cycle arrest rather than inhibition of DNA-pol alpha or Cdk.
The experiment where the authors separate leading from lagging strand telomeres and analyze overhang length by in gel hybridization answers the criticism from reviewer 2 that the authors did not directly show that leading strand telomeres are processed. The authors now demonstrate that overhangs are indeed present on the leading strand telomeres so they must have been processed. They do not answer the question as to whether the terminal sequence of the leading strand telomeres is maintained after processing. This is a pity, but understandable given the technical problems. However, what is disturbing is that the results differ from those obtained from a similar (but not identical) experiment published in the Zhao 2009 Cell paper. Zhao et al report a net increase in overhang length at leading strand telomeres during S-phase while the authors of the current manuscript report a decrease in length. While the timing of the analysis done by Zhao et al. was somewhat different, it is very difficult to reconcile the results from the two labs. Unfortunately, the authors of the current manuscript simply ignore this discrepancy. Clearly it was an unexpected result and one which they do not appear to have come to grips with as they mention telomerase extension of leading strand telomeres in the discussion on page 16 (line 9) but yet say elsewhere that their results indicate that this does not occur.

Our interpretation of Zhao et al.'s data differs from that of the reviewer. In Zhao et al.'s study, leading overhangs were measured at only two time points and with different labeling methods. One was a 30 min pulse BrdU labeling at 3 hrs after release, while the other was continuous BrdU labeling for 8 hrs throughout S phase. The authors showed that leading overhangs were ~70 nt shortly after replication and then barely decreased to ~63nt after telomeres completed replication. As there was no statistical analysis in the study, we do not know the statistical significance of the 7nt decrease. However, during the same time period, lagging overhangs decreased from 100 nt to 60 nt, suggesting that lagging telomeres were filled in for ~40 nt. If similar C-strand fill-in also occurred at leading telomeres, the leading overhangs should have been decreased to ~33 nt (=73 ñ 40). The fact that leading overhangs only decreased by 7 nt (if it is statistically significant) suggest that C-strand fill-in probably was absent from leading telomeres. In fact, throughout the paper, the authors focused on lagging telomeres and did not claim that C-strand fill-in also occurred at leading telomeres.
Although Zhao et al. pointed out that there were more short overhangs at leading telomeres 8 hrs after release than those from telomeres replicated for just 30 min, we think that this might have been caused by the limitation of the DSN assay used for measuring very short overhangs in Zhao et al.'s study. In the DSN assay, very short overhangs (<21 nt) and blunt-ended telomeres are excluded. In the 30 min pulse labeled sample, due to such a short labeling time, a great proportion of telomeres just finished replication and would not have enough time to be processed and extended by telomerase. This population of leading telomeres would have contained extremely short or no overhangs and could not be detected by the DSN assay. But these telomeres could undergo end resection in G2 and their overhangs then became measurable by the DSN assay. These resected telomeres could have contributed to the increase in the fractions of short overhangs at 8 hrs after release. We also notice that in Zhao el al.'s study the fraction of very long overhangs (>384 nt) increased at 8 hrs after release (Fig. 3E in Zhao et al.). Unfortunately, the authors ignored this increase and only pointed out the increase in the fraction of short overhangs. Since the analysis of leading overhangs in Zhao et al. was limited to 8 hrs after release, we believe that if the authors had measured the leading overhangs at 10 hrs after release, probably an increase in leading overhang length would have been detected.
In our study, we intentionally used the non-denaturing in-gel hybridization assay to capture overhang signals from all of the telomeres. Although the drawback of this assay is that it does not measure the absolute length of overhangs, it does include telomeres with blunt ends and with very short overhangs.
From above analyses, we think that it is difficult to compare our data in leading overhangs with Zhao et al.'s results. The differences are that (1) different methods were used; (2) there are only two time points in Zhao et al.'s study; and (3) we measured overhangs in G2 phase after replication has completed, while Zhao et al. did not.

The type of analysis described in this manuscript and by Zhao et al. is technically very difficult and prone to artifacts. Thus, there is currently no way to tell which lab is correct. For this reason, I would recommend leaving the leading versus lagging strand telomere analysis as part of the paper. However, I think the authors should address the discrepancy with the Zhao results and tone down their conclusions.

We agree with the reviewer that it is difficult to compare our data in leading overhangs with previously published results due to reasons mentioned above. We have provided the following discussion to address the discrepancy on page 18-19.
"Fig. 3D shows that leading telomeres possess overhangs throughout S phase, in agreement with the previous report that the blunt-ended leading telomeres are processed immediately following replication to yield overhangs in order for telomerase to extend leading telomeres (Zhao et al, 2009). Interestingly, we found that while lagging overhangs were elongated during S phase and then shortened in late S/G2 by C-strand fill-in, leading overhangs remained stable during S phase and were further extended in G2/M phase (Fig. 3D), indicating that the late S/G2- specific C-strand fill-in may be absent at leading telomeres. Although it seems surprising, Zhao et al. also reports that the lengths of leading overhangs remain largely unchanged from 30 min after replication to late S/G2 phase (Zhao et al, 2009). The authors' measurement of leading overhangs in S phase was ~70 nt, while in late S/G2 phase the length of leading overhangs was barely diminished to ~63 nt. During the same time period, lagging overhangs shortened from 100 nt to 60 nt (Zhao et al, 2009), indicating that C-strand fill-in had occurred at lagging telomeres. If similar C-strand fill-in also occurred at leading telomeres, the leading overhangs should have been decreased to ~33 nt (=73 ñ 40). Taken together, these observations support the notion that following telomerase elongation of leading telomeres, the sizes of leading overhangs remain largely constant during S phase and C-strand fill-in is likely to take place at lagging telomeres but not at leading telomeres. Although there is a great discrepancy in the amount of G-overhangs measured from the two studies, this is probably caused by differences in methods used for measuring G-overhangs. The present study uses non-denaturing in-gel hybridization assay, which measures the relative abundance of ss overhangs from all of the leading telomeres including the ones with blunt ends or with extremely short overhangs. The drawback of this assay is that it does not measure the absolute length of overhangs. In contrast, the DSN assay used in the previous study is able to measure the length of overhangs but excludes telomeres with blunt ends or with extremely short overhangs. Therefore, it is likely that the length of leading overhangs measured by Zhao et al. was an overestimation.
Analysis of the dynamics of leading overhangs in the previous study was limited to 8 hrs after release, leaving the question of what happens to leading telomeres at later time points after cells enter G2 phase (Zhao et al, 2009). In the present study, we observed an unexpected increase of leading overhangs in G2/M (Fig. 3D), suggesting that additional enzymatic events might have acted on leading telomeres after replication. The G2/M-specific elongation of leading overhangs could be due to a second processing event. In agreement with this view, it has been shown that nucleases involved in DSB processing are recruited to telomeres during the G2 phase of the cell cycle (Verdun et al, 2005). Our findings further stress the need to identify nucleases and helicases responsible for the two processing steps at leading telomeres. Multiple nucleases and helicases that have been identified in budding yeast, including ExoI, Dna2, Sgs1, Sae2, and the Mre11/Rad50/Xrs2 complex, that contribute to C-strand resection (Bonetti et al, 2009). It remains to be determined whether similar nucleases/helicases are involved in processing telomere ends in human cells. An alternative explanation for the G2/M-specific lengthening of leading overhangs is that telomerase might extend leading G-strands at G2. This is considered less likely, as telomerase is recruited to telomeres during S phase (Tomlinson et al, 2006) and extends the majority of telomeres during S phase (Zhao et al, 2009)."

Another concern revolves around the author's conclusion that the DNA damage response caused by the purvalanol treatment results from the accumulation of single-stranded G-strand DNA. The actual increase in G-overhang is fairly modest (<50%) so there may well be enough Pot1 (or CST) in the cell to coat the extra overhang and exclude RPA. An alternative reason for the damage response might be that the purvalanol treatment also inhibits processes needed to resolve other replication defects. These could include replication fork stalling in the telomeric tract which would then result in telomeric gamma H2Ax foci.

The reviewer is correct that we cannot make the conclusion that the increased gamma H2AX in purvalano treated cells is due to the accumulation of ss G-rich DNA. We have modified the results section and added the following clarification to the manuscript on page 14-15:
"The elevated telomeric DNA damage upon CDK1 inhibition might have resulted from the accumulation of ss G-rich DNA. However, a significant proportion of normal human telomeres contain long overhangs that are protected from activating DNA damage response. Given that the observed G-overhang increase is <50% when CDK1 is inhibited, there still might be an adequate amount of ssDNA binding protein such as Pot1 or CST to protect most elongated overhangs. The residual elongated overhangs may be insufficient to trigger massive telomeric DNA damage response. Indeed, the number of telomeric -H2AX foci is fairly modest compared to that induced by dysfunction of shelterin proteins (Denchi & de Lange, 2007; Konishi & de Lange, 2008; Sfeir et al, 2009; Takai et al, 2003), indicating that only a proportion of telomeres became dysfunctional. Alternatively, CDK1 activity may be needed for resolving other replication defects such as replication fork stalling at telomeres by an unidentified mechanism, which might, for example, involve post-translational modification of the shelterin complex."
In addition, we deleted the last paragraph on p18-19 in the original manuscript (revised page 20), since at present it is unclear whether the telomeric DNA damage response is indeed caused by elongation of overhangs. The subtitle has been changed from "The critical role of G-overhang generation in DNA damage response at telomeres" to "Protection of elongated ss G-rich DNA".

Other comments:
Page 8, line 18. The extended G-overhangs could be due to telomerase extension in addition to C-strand resection as the experiment was done in HeLa cells. To draw a conclusion that the extended G-overhangs in S-phase were due to extensive resection, the experiment would have to be repeated in telomerase negative cells.

The reviewer is correct, since the majority of our experiments were carried out in HeLa. We have modified the text throughout the manuscript to include telomerase extension in addition to C-strand resection when discussing about G-overhang elongation.

Page 17, line 5. Remove "first" as other work has also provided insights into G-overhang generation at leading and lagging daughter telomeres.

It has been deleted.

Legend to Figure 2 B. Remove "comparing to DMSO" (page 28, line 4) as this is confusing. The FACS profile is shown beside (not below) the G-overhang analysis.

We deleted it.

