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RPA facilitates telomerase activity at chromosome ends in budding and fission yeasts

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1st Editorial Decision 06 July 2011

We have now received a complete sets of comments on your RPA manuscript. All three referees raise a number of (partially overlapping) major concerns, but would in principle support publication in The EMBO Journal should you be able to adequately address these points. Given that their point seem to be well taken and that they pertain mostly to the current data set (and not to a major extension of the study beyond its scope), I would like to give you the opportunity to answer and address the various issues in the form of a revised version of the manuscript. Should you be able to satisfactorily improve the study along the lines raised in the referees' comments, then we should be happy to consider the study further for publication. Please note however that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Therefore, should you have any concerns regarding the experiments requested by the reviewers, I would encourage you to get back to me for further consultations.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For

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http://www.nature.com/emboj/about/process.html

Should you have any additional question regarding this decision or your revision, please do not hesitate to contact me. I look forward to your revision.

With best regards,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript examines the role of RPA complex in telomere replication and its interactions with telomerase holoenzyme in budding and in fission yeasts. Co-immunoprecipitation experiments reveal that RPA physically interacts with telomerase in both systems. In case of budding yeast RPA, the authors went further to show that this interaction is mediated through both yKu and Est1 proteins. The data confirms previous findings by the same group that RPA complex associates with telomeres at the time of their replication and further demonstrate that this association occurs at both leading and lagging telomere strands. It is also shown that the RPA association with the leading strand requires Mre11, as does its interaction with telomerase. A specific allele of RFA1, encoding the largest RPA subunit, is defective in its interaction with telomerase. Although this allele itself does not affect telomere length in budding yeast, it limits the overelongation in cells lacking Rif proteins, thus supporting the notion that RPA-telomerase interaction positively regulates telomerase activity. Moreover, the role of this interaction seems to be conserved since similar reduction of telomere length is seen in S.pombe strains lacking negative regulators of telomerase.
In my opinion the reported observations on RPA-telomerase interactions are novel and help to better define the RPA function at telomeres. Nonetheless, the following issues have to be addressed to unequivocally support the conclusions drawn by the authors. There are also certain technical issues that preclude publication of this manuscript in its present form.

Specific comments:

1) Based on their findings, the authors propose a model in which RPA interacts with and stimulates telomerase specifically at the leading strand telomere. Although Mre11 appears to play a decisive role in those events, I am rather puzzled about its mode of action and the order of those events. First, is the role of Mre11 limited to C-strand resection and ssDNA generation for RPA binding? Since Mre11 only associates with and is responsible for resection at the leading telomere strand (Faure, 20101), why would Mre11 deletion almost completely abolish RPA-telomerase interaction instead of reducing it in half? The ssDNA, RPA and telomerase are all present at the lagging strand in mre11 mutants so, the RPA-TLC1 interaction could in principle still occur there. Would the absence of Mre11 itself rather than its resection activity preclude the RPA-telomerase interaction at lagging telomeres and could the authors use specific Mre11 mutants to better define the function of Mre11? Do the RPA telomere association and its interaction with telomerase increase in absence of Rif proteins given that Mre11 access to telomeres is increased in the rif mutants? Second, the RPA-TLC1 interaction depends apparently on wild-type levels of telomerase at the leading telomere since both Est1- and yKu-dependant recruitment pathways affect this interaction. As competition between RPA and Cdc13p for resected telomeric ends is expected to occur, I wonder if Cdc13 binding precedes RPA binding and how it would influence RPA-telomerase interaction. Can the authors employ cdc13-2 and est1-60 mutant strains to address this question?

2) One serious issue with this work is that the nature of BrdU-labaled DNA in Rpa1 ChIPs (Fig.1) is not rigorously controlled for the presence contaminating non-telomeric genomic DNA. As oppose to Cdc13p, which binds specifically telomeric DNA in sequence-specific manner, RPA complex can associate with any single-stranded genomic region. The Y'-probed blot may be appropriate to analyse Cdc13p ChIP samples but is not stringent enough as a control for RPA ChIPs. The authors

should carry out hybridization of these filters with a random genomic probe, ideally before and after LM-PCR amplification of ChIP DNA, to rule out contribution of contaminating genomic DNA in BrdU signals. Actually, it is rather surprising to see BrdU signals after Rfa1 ChIP down at zero during the first cell cycle in Fig. 1E as one would expect to find some BrdU-labeled genomic DNA associated with RPA throughout the S phase in replicating cells.

3) Again in Fig.1, why is the BrdU incorporation in non-telomeric DNA (bulk genomic DNA) 7 fold lower in mre11 cells as compared to WT cells? Moreover, given that the length of telomeric repeats in mre11 cells is at about 1/3 of the wild type length, this would be expected to reduce further the level of telomere-incorporated BrdU with approximately 20 fold. This is hard to reconcile with barely 2 fold reduction of ChIP BrdU signals in mre11 samples in Fig. 1(compare WT and mre11 graphs for total and second cell cycle incorporation in Fig.1D and 1E).

4) The statement on page 6 that Cdc13p binding is slightly reduced in mre11 cells (Fig.1B) should be softened. The decrease is significant, perhaps 60-70% of WT level, and the reduction for Rpa is slightly bigger (around 50%); so, the difference of reduction between Cdc13 and Rpa1 in this background is not that dramatic as one may get impression from the author's description. In addition, the synchronization is not exactly the same between Wt and mre11 cultures and, therefore, it may not be entirely appropriate to compare directly the 45min time point for the two backgrounds. Likewise, the statement on page 7 that the presence of RPA on the leading strand telomere is at least partially dependent on Mre11 does not stand right along with the complete absence of ChIP BrdU signals in Fig. 1E.

5) The authors should also correct their conclusion on page 9 that RPA and yKu interact with each other preferentially at the end of S-phase because it is not supported by the data. The time course experiment in Fig. 3C and 3D actually shows that RPA and yKu interact throughout the cell cycle, except in G1, including at 30min and 75min after release when RPA is not enriched at telomeres.

6) Some of the data on the RPA-yKu interaction, specifically panels 3C, 3D and 5B, could be moved to the Supplemental data section because of the above reasoning and because the reduced rfa1D228Y-yKu interaction arguably may stem from reduced binding of rfa1D228Y to telomeric DNA, not yKu (see below).

7) The observation that rfa1-D228Y mutant is partially defective in telomere association is rather surprising considering the crystal structure model presented in figure S3 and in the light of the fact that the equivalent rad11-D223Y mutation in fission yeast does not seem to affect DNA binding. Is the rfa1-D288Y mutant protein proficient in DNA binding? Or the decreased telomere association in Fig. 5A results only from defective interaction with other telomere-bound protein partners? Is the replication affected in rfa1-D288Y cells?

8) The authors should carefully verify the manuscript for mislabelled figures and mistakes in figures citations in the text. Examples: in the legend of Fig.1, (C) and (D) do not correspond to the figure panels, (E) is missing in the legend but present in the figure; in first paragraph on page 15, in the brackets, 5A should be 4A... They also should take care to describe more clearly the methodology in Methods and in figure legends to facilitate understanding. For instance, in Fig.1 it is unclear the telomeres on which chromosome were analysed after ChIP (none of the 3 primer pairs listed in Methods corresponds to the chromosome specified in the figure legend) and which genomic locus was used as a control to calculate the fold enrichment (in Methods they point Gal2 but no primers shown in the Table S2; instead primers for ARO1 and ADH4 listed - why?). Also, the concentration of thymidine competitor added during the second cell cycle cannot be 2000 g/ml.

Referee #2 (Remarks to the Author):

The authors previously reported that budding yeast RPA (replication protein A) associates with telomeres and regulates the telomerase reaction through recruiting Est1 to telomeres (Nat genet, 2004). In this manuscript, they propose that RPA associates with Ku proteins and that telomeric RPA recruits telomerase via the RPA-Ku and RPA-Est1 interactions. Overall, experimental data supports the authors'conclusion. However, I have technical concerns as described below.

Major concerns
1. Quantification of Chip data
I do not understand the rationale to quantify the Chip results by "the relative values of bound telomeric DNA (TelVI-R) over background" (Fig. 1, legend). The authors do not indicate what sequences they used in "background" measurements. If they measured the amount of non-telomeric DNA sequences in Cdc13-Flag or RPA-ChIP, the values are theoretically close to zero. In this case, "the relative values" should be very large and do not make sense as a measure because even small stochastic fluctuation of the background value (around zero) will produce a tremendous fluctuation of the relative value.

2. The authors' conclusion that RPA physically associates with telomerase is based on a) detection of TLC1 RNA in RT-PCR experiments of Rfa1 IP; b) TLC1-RPA interactions detected by co-IP/RT-PCR showed similar changes during the cell cycle as those of TLC1-tel and RPA1-tel associations; c) TLC1-RPA interactions detected by co-IP/RT-PCR are additively defective in tlc1-delta48 and est1-delta mutation; d) TLC1 was not detected in RPA ppt in the Rfa1-D228Y background; and e) Telomere elongation in rif1-delta and/or rif2-delta was rescued the Rfa1-D228Y background.

Although all experimental data are supportive for the RPA-telomerase interaction, most are indirect evidence. Because RPA is an abundant protein in cells, authors need carefully exclude artificial associations. Authors briefly mentioned that they could not detect Est2 in RPA ppt. Did they try to detect telomerase activity coIP'ed with RPA to show that RPA associates with active telomerase holoenzyme?

3. The authors' conclusion that RPA physically associates with Ku is based on a) reciprocal IP-IB experiments of Rfa1 and yKu80-myc; b) RPA-yKu interactions detected by co-IP showed similar changes during the cell cycle as those of yKu80-tel association; and c) TLC1-RPA interactions detected by co-IP/RT-PCR are Mre11-dependent as in the cases of TLC1-tel and RPA1-tel associations.

Fig. 2A: Co-immunoprecipitation experiments of Rfa2 in Fig. 2A are difficult to interpret. The Rfa2 bands are closely located with other intense bands (presumably derived from Ig used for IP) and the specificity is not clear. Experiments using anti-Rfa1 should give clearer results as shown for Rfa1-Ku interactions in Fig. 3A.

Fig. 3A
What is the yKu80-myc signal in lane 3 of the left panel (anti-myc IP of untagged control cells)?

Fig. 3C
It appears to me that the migration of Rfa1 coIP'ed with yKu80-myc is slightly slower than Rf11 in the input (lower panel). If this is the case, what caused the difference? What is the fast migrating band in yKu80-myc IP fractions (upper panel)?

Minor concerns
1. Fig. 1
The legends are not consistent with the actual figure, although the text refers to the correct figures. In the legends, they say (B) indicates Chip results of Cdc13-Flag and RPA, but in the actual figures they are presented in (B) and (C), respectively. In the legends, they say BrdU Chip results of wt and mre11-D cells are shown in (C) and (D), but in the figures they are shown in (D) and (E).

2. Page 13, "Rad3 that leads to a substantial telomere shortening (Nakamura et al, 2002; Kanoh et al. 2003)"
These citations are inappropriate. The first paper showing the Rad3-deletion results in substantial telomere shortening was Naito et al. Nat. Genet. 1998.

Referee #3 (Remarks to the Author):

The molecular steps involved in regulating telomere elongation have focused to a large extent on understanding the interplay of telomere-bound proteins with telomerase. How more general DNA

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replication proteins such as RPA fit into the picture has not been entirely clear. This manuscript shows that RPA can interact with both yKu80 and the telomerase RNA, and requires Mre11 function to efficiently bind to the leading strand telomere. The model constructed from the data is that RPA binds to resected telomeres, then transiently interacts with telomerase that is present. That Mre11 function promotes RPA leading-strand telomere association is certainly consistent with RPA binding to the 3' single-strand telomere DNA following resection, as opposed to being enriched due to the replication fork moving through or pausing at telomeres. A conserved, RPA-dependent telomere length regulation mechanism is proposed to exist, based on evidence from both S. cerevisiae and S. pombe yeast species. Given the conservation in the interaction between RPA and telomerase, it would be interesting to follow this data with an investigation into whether the RPA association with leading strand telomeres is affected by telomerase, or conversely whether telomerase association on the leading strand is adversely affected by loss of RPA interaction.

Major points

1. The data in Figure 1 strongly indicate that Mre11 positively influences RPA recruitment to leading but not lagging strand telomeres. This is an important finding. Since RPA is not telomere specific in its DNA binding, it is curious that no BrdU was detected in the Rfa1 ChIP in the first cycle after BrdU exposure. Is the crosslinking at other genomic locations far less efficient?

2. Since telomeres in mre11 strains are short, it is important to know whether the reduced telomere length accounts for the lower enrichment of telomere fragments in the ChIP data for figure 1. It was previously reported (by some of these authors) that if one normalizes to telomere TG1-3 sequences in the analysis of Cdc13 ChIP, much of the difference in ChIP efficiency between MRE11 and mre11 cells goes away. However, in figure 1, TelVI-R was used to measure the telomere DNA in the ChIP. This probe will not take into account the difference in telomere length between the strains. Therefore, in the data shown, it is difficult to separate the impact of telomere length versus Mre11 absence on RPA telomere association.

3. Some conclusions the authors make in their comparisons of Cdc13 and Rfa1 telomere associations are not as well substantiated by the data as stated in the text. The data comparing Cdc13 (here and from Faure et al 2010) and Rfa1 telomere localization seems to be in fact more similar than different. As discussed further below, RPA does not necessarily seem to be more sensitive to Mre11 function than Cdc13 is. Instead, it seems that the presence of both Cdc13 and RPA on leading strand telomeres is affected by Mre11.

The authors conclude from the data in figure 1 that loss of Mre11 has more of an impact on Rfa1 ChIP than on Cdc13-Flag ChIP, saying Cdc13 ChIP is only slightly reduced in mre11 whereas RPA ChIP is reduced by 2x. However, the data shown do not necessarily support this conclusion. From the graphs shown in 1B and 1C, it looks like the TelVI-R enrichment measurement for Cdc13 ChIP (at 45 minutes) is ~ 23/33 in mre11 / MRE11, and for Rfa1 is ~20/33 in mre11/ MRE11. The difference is more pronounced in the Rfa1 data from 1D and 1E. Cdc13 is not shown for comparison here, although such Cdc13 data was published in the Faure et al 2010 paper.

Whether or not Mre11 affects RPA more than Cdc13 is really a minor point for this paper. Instead of trying to include further experiments to better substantiate a difference, it may be better to be more careful in the comments in the text.

3. The cell cycle timing of Cdc13 and RPA telomere association seems to vary substantially when comparing the data in figure 1, even though the figure legend seems to suggest that these data came from the same cell cycle release experiments. Why is the peak of Cdc13-Flag and Rfa1 telomere association 45 minutes in 1B, 1C and 5A but in 1D and 1E the peak for Rfa1 it is 70 minutes? Does this mean the peak of Cdc13 and RPA telomere binding is before the region is replicated? Is the 45 minute time point really late S phase? If the legend was clearer, it may help to resolve this issue.

4. The biochemical data indicates that RPA can co-immunoprecipitate with yKu80 and TLC1. Both Rfa1 and Rfa2 were tested, and the data are strongly substantiate these associations. However, the data in Figure 2A testing interaction of Rfa2 with Cdc13 and Est2 is not convincing. The Rfa2 band is quite difficult to see, due in part to the close migration of the antibody chain. Why not use the anti-Rfa1 antibodies for these co-IPs? Alternatively, you could avoid the IgG bands by using a

chicken anti-myc antibody for the IP.

Minor points

4. The figure legend (A-D) for Figure 1 does not completely correspond with the figure displayed (A-E?). A better written legend would be quite helpful.

5. What do bars represent in ChIP figures? Error in measurement for one experiment? Standard deviation among multiple biological replicates? It is important to include this information in the paper.

6. On page 7, toward the end of the first paragraph, it is written that BrdU signal was associated with Rfa1-ChIP at 50 and 70 min after the G1-releases. However, the data in Figure 1 looks like the peak associations are at 70 min for both cycles.

7. On page 9, the last sentence in the middle paragraph states that RPA and yKU interact with one another preferentially at the end of S phase when both are at telomeres. This seems a bit strong, and could just be qualified a bit, as was done in the discussion. (Is the amount of Rfa1 in the yKu80-myc IP really greater at 45 minutes? yKu80 and Rfa1 are clearly seen to co-IP at 30 minutes, when RPA is not strongly crosslinked with telomere DNA. It is possible these interactions take place elsewhere in the genome or independent of DNA..)

8. On page 18, in the middle paragraph, it should say that RPA stimulates the elongation of the G-strand or 3' strand by telomerase - not the 5' strand.

1st Revision - authors' response 19 December 2011

We are grateful to all referees for their insightful comments, which helped us to improve the manuscript.

All the changes that we have made appear in red in the revised version and in the supplemental information and therefore can be easily visualised.

To answer referee 1 (points 1 to 4), referee 2 (point 1) and referee 3 (points 1 to 3) (further below we address each point individually), we have performed the ChIP/BrdU assay shown in the initial version in Fig. 1D and 1E with the two following changes : i) To improve the sensitivity of the ChIP/BrdU assay, the experiments were performed in WT and *mre11*Δ cells expressing a tagged version of Rfa1 (Rfa1-myc18) and able to efficiently incorporate BrdU, and ii) Binding of Rfa1-Myc18 to telomere and to a control genomic region in WT and *mre11*Δ cells has been analyzed by hybridization with radiolabelled telomeric TG_{1-3} and GAL2 probes, respectively.

A strain W303-1A (*MAT a*, *RFA1-MYC18::TRP1*) was crossed with W303-1B (*MAT alpha, bar1::TRP1 RAD5, URA3-GPD-TK, phENT1-LEU2*). From the dissection of the diploid, *w*e isolate a spore PL9T163 with the following genotype (MAT a, *bar1::TRP1, RFA1-myc18::TRP1, ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11,15, ura3-1, GAL, RAD5+, URA3 GPD-TK, phENT1-LEU2*). This new strain (PL9T163) expresses a functional Rfa1-Myc18, efficiently incorporates BrdU and exits from the alpha-factor block more efficiently than the PP529 strain used in the original version. We then disrupted *MRE11* in PL9T163 to give PL9T163 *mre11::HIS3*.

We have used PL9T163 and PL9T163 *mre11::HIS3* to perform new ChIP/BrdU experiments similar to those described in Fig. 1D and 1E of the original submission. As shown in the new Fig. 1D, E, (and the new Fig. S1) (see below), the ChIPs were analyzed both by qPCR and by hybridization with a TG_{1-3} probe or a genomic GAL2 probe. Compared to the original PP529 strain, the new PL9T163 enters S-phase after α-factor release more efficiently. With both detection methods (qPCR and hybridization), for the PL9T163 strain, RPA telomere association during the two consecutive cell cycles is observed at 30 min after α-factor release, reaches its maximum at 40 min and strongly

decreases at 50 min. For the PL9T163 *mre11::HIS3,* telomere binding starts at 40 min and reaches the maximum at 50 min in agreement with the cell cycle delay of the *mre11*Δ cells. In both strains, no hybridization signal was observed at any of the time point when the membrane was hybridized with the GAL2 probe. Probably the time of residence of replicating RPA is too short at the *GAL2* locus to give a detectable signal. Note that studies aimed to ChIP RPA at regions adjacent to replication origins are performed in the presence of hydroxyurea (HU), which causes replication fork stalling and accumulation of ssDNA.
Interestingly, binding of RPA to telomeres in late S-phase was similar in WT and *mre11*Δ cells when the RPA ChIPs were analyzed by hybridization with the radiolabelled TG_{1-3} and normalized to the input DNAs. As mentioned by referee 3, because telomeres in the *mre11*Δ strain are short, it was important to know whether the reduced telomere length accounts for the lower enrichment of telomere fragments in the ChIP experiments. Indeed, we observed previously (Faure et al. 2010) that about the same proportion of telomeric DNA were precipitated with Cdc13 in WT and *mre11* cells when the ChIPs were analyzed by hybridization with radiolabelled TG_{1-3} probe. Similarly, we find now that the amount of telomeric DNA precipitated with RPA normalized to total telomeric is similar in WT and *mre11*Δ cells.
Therefore, we have softened throughout the revised version of the MS our statement that RPA telomere binding is dependent on Mre11. It still holds though that the interaction between RPA and telomerase RNA component (TLC1) strongly depends on Mre11. This means that only a fraction of telomere-bound RPA interacts with telomerase.

The BrdU signals were then analyzed as described in the original submission. Again, BrdU signals were mainly obtained for the time points when RPA binds to telomeres. As in the original submission, in WT cells (PL9T163) BrdU was detected for the two consecutive cell cycles while for PL9T163 *mre11::HIS3* cells BrdU signals were strongly reduced during the first cell cycle but not during the second cell cycle. Therefore, in *mre11* cells, RPA telomere binding at the leading strand (that accounts for 30% of the total RPA in WT cells) is strongly decreased. Since we do not observe a significant reduction of the total amount of telomere-bound RPA, this result indicates that a large fraction of telomere-associated RPA that we detect by ChIP is the "replicative" RPA that binds at the lagging strand telomere. It is likely that both the time of residence and the amount of RPA is higher at the lagging strand than at the leading strand consistent with the fact that in WT cells the amount of BrdU associated with the RPA-ChIP is higher for the second cell cycle.
It is important to point out that the signal revealed with anti-BrdU antibodies in ChIP samples is very weak and is only detectable when the amount of precipitated DNA exceeds a certain threshold. Therefore, the fact that we obtain no BrdU signal in the ChIP does not mean that DNA is absent from the ChIP, as the case with the *mre11*Δ cells, which incorporate BrdU less efficiently. Actually, we have observed that total incorporation of BrdU is decreased by at least two fold in *mre11*Δ cells (not shown). Therefore, if the amount of DNA in the RPA ChIP during the course of replication is too low, it is likely that it will be undetected by the anti-BrdU antibodies.

These changes have been incorporated in the revised version (new Fig. 1 D, E) and new Fig. S1

Referee #1 (Remarks to the Author):

The manuscript examines the role of RPA complex in telomere replication and its interactions with telomerase holoenzyme in budding and in fission yeasts. Co-immunoprecipitation experiments reveal that RPA physically interacts with telomerase in both systems. In case of budding yeast RPA, the authors went further to show that this interaction is mediated through both yKu and Est1 proteins. The data confirms previous findings by the same group that RPA complex associates with telomeres at the time of their replication and further demonstrate that this association occurs at both leading and lagging telomere strands. It is also shown that the RPA association with the leading strand requires Mre11, as does its interaction with telomerase. A specific allele of RFA1, encoding the largest RPA subunit, is defective in its interaction with telomerase. Although this allele itself does not affect telomere length in budding yeast, it limits the overelongation in cells lacking Rif proteins thus supporting the notion that RPA-telomerase interaction positively regulates telomerase activity. Moreover, the role of this interaction seems to be conserved since similar reduction of telomere length is seen in S.pombe strains lacking negative regulators of telomerase.
In my opinion the reported observations on RPA-telomerase interactions are novel and help to better define the RPA function at telomeres. Nonetheless, the following issues have to be addressed

to unequivocally support the conclusions drawn by the authors. There are also certain technical issues that preclude publication of this manuscript in its present form.

Specific comments

1) *Based on their findings, the authors propose a model in which RPA interacts with and stimulates telomerase specifically at the leading strand telomere. Although Mre11 appears to play a decisive role in those events, I am rather puzzled about its mode of action and the order of those events. First, is the role of Mre11 limited to C-strand resection and ssDNA generation for RPA binding? Since Mre11 only associates with and is responsible for resection at the leading telomere strand (Faure, 2010), why would Mre11 deletion almost completely abolish RPA-telomerase interaction instead of reducing it in half? The ssDNA, RPA and telomerase are all present at the lagging strand in mre11 mutants so, the RPA-TLC1 interaction could in principle still occur there. Would the absence of Mre11 itself rather than its resection activity preclude the RPA-telomerase interaction at lagging telomeres and could the authors use specific Mre11 mutants to better define the function of Mre11?*

As mentioned above, 70% of RPA that we detect at telomeres by ChIP is localized at the lagging strand telomere. The fraction of RPA at the leading strand telomere (30%) is sensitive to Mre11 and may be linked to ssDNA generation. We agree with referee1 that nearly complete loss of RPA-telomerase interaction after Mre11 deletion is most likely due to the Mre11 role in recruiting telomerase to telomeres rather than to its role in resection. We also agree that one cannot exclude that the absence of Mre11 precludes the RPA-telomerase interaction at lagging telomeres. The article has been amended accordingly. We have started to study specific alleles of Mre11 but this represents a whole separate study.

2) One serious issue with this work is that the nature of BrdU-labaled DNA in Rpa1 ChIPs (Fig.1) is not rigorously controlled for the presence contaminating non-telomeric genomic DNA. As oppose to Cdc13p, which binds specifically telomeric DNA in sequence-specific manner, RPA complex can associate with any single-stranded genomic region. The Y'-probed blot may be appropriate to analyse Cdc13p ChIP samples but is not stringent enough as a control for RPA ChIPs. The authors should carry out hybridization of these filters with a random genomic probe, ideally before and after LM-PCR amplification of ChIP DNA, to rule out contribution of contaminating genomic DNA in BrdU signals. Actually, it is rather surprising to see BrdU signals after Rfa1 ChIP down at zero during the first cell cycle in Fig. 1E as one would expect to find some BrdU-labeled genomic DNA associated with RPA throughout the S phase in replicating cells.

As mentioned above, BrdU signals are weak and only detectable when the amount of DNA bound to the ChIP exceeds a threshold. Therefore, the fact that we obtain no BrdU signal in the ChIP does not mean that DNA is absent from the ChIP, particularly when BrdU incorporation is less efficient. This may explain why we see BrdU signals after Rfa1 ChIP down to zero during the first cell cycle in mre11 cells (Fig. 1E of the first submission). As shown above, in the new Fig. 1D, E we have carried out hybridization of the ChIPs filters with a GAL2 probe. The results suggest that the amount of non-telomeric genomic DNA associated with the RPA-ChIP is minor compared to the amount of telomeric DNA.

3) Do the RPA telomere association and its interaction with telomerase increase in absence of Rif proteins given that Mre11 access to telomeres is increased in the rif mutants?

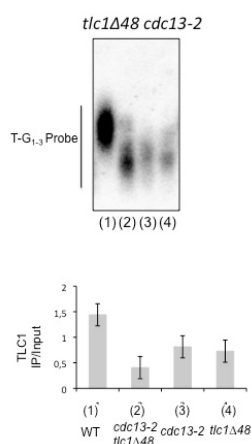
As suggested by referee 1, we have analyzed the binding of RPA and Cdc13 in the absence of Rif1 and Rif2 (see below). Binding of RPA and Cdc13 were analyzed by ChIP followed by hybridization with a TG_{1-3} probe since telomeres are over-elongated in a *rif1*Δ *rif2*Δ double mutant. The results show that the total amount of Cdc13 bound to telomeres is similar in WT and *rif1*Δ *rif2*Δ cells. This means that when the level of telomere-bound Cdc13 is normalized to the total amount of TG_{1-3} sequences (input DNA), the level of Cdc13 drops by a factor 4. For RPA, its telomere binding increases in the *rif1*Δ *rif2*Δ double mutant and therefore the level of telomere-bound RPA normalized to the total amount of telomeric sequences decreases by only a factor 2.These results suggest that Cdc13 is present mainly at the end of the telomeres and does not seem to act as a replicative telomeric RPA (t-RPA). The fact that the amount of telomere-bound RPA increases may be related to its replicative role or to its role in stimulating telomerase activity. We have then

measured the interaction between RPA and telomerase. We observed an increase (30%) of the association between RPA with TLC1 in the *rif1Δ rif2Δ* double mutant that could reflect the increase accessibility of telomerase to telomeres. Given the slight increase of the interaction between RPA and TLC1, we prefer to be cautious about this statement.

This figure will be added as a supplemental figure related to Fig. 5.

4) Second, the RPA-TLC1 interaction depends apparently on wild-type levels of telomerase at the leading telomere since both Est1- and yKu-dependant recruitment pathways affect this interaction. As competition between RPA and Cdc13p for resected telomeric ends is expected to occur, I wonder if Cdc13 binding precedes RPA binding and how it would influence RPA-telomerase interaction. Can the authors employ cdc13-2 and est1-60 mutant strains to address this question?

From the many ChIP experiments that we have done, we just can say that telomere binding of RPA and Cdc13 always peaks at the same time point.
We have analyzed the interaction of RPA with TLC1 in a *cdc13-2* mutant in the same conditions as those described in Fig. 4B. We observed that the *cdc13-2* allele reduces the interaction of RPA to telomerase as does the deletion of *EST1*, strengthening the results of Fig. 4B. However, when the *cdc13-2* allele is combined with the *tlc1Δ48* allele, the RPA/TLC1 interaction is not completely abolished in contrast to what happens to the *tlc1Δ48 est1Δ* double mutant. Although this latter result is consistent with the recent observation from Wu and Zakian (PNAS, 2011) showing a week but significant direct interaction between RPA and Est1, and with the fact that Est1 interacts as well with Cdc13 or Cdc13^{E525K} (*cdc13-2*), for a sake of clarity, we would prefer to leave out these data from the MS.



Diploids Mata/Mata tlc1Δ48/TLC1 cdc13-2/CDC13 cells were sporulated and telomere lengths of the 4 spores from a tetratype were analysed by Southern blot using a TG_{1-3} probe (Top panel). RPA/TLC1 interaction was analyzed for each spore of the tetratype (low panel).

5) Again in Fig.1, why is the BrdU incorporation in non-telomeric DNA (bulk genomic DNA) 7 fold lower in mre11 cells as compared to WT cells? Moreover, given that the length of telomeric repeats in mre11 cells is at about 1/3 of the wild type length, this would be expected to reduce further the level of telomere-incorporated BrdU with approximately 20 fold. This is hard to reconcile with barely 2 fold reduction of ChIP BrdU signals in mre11 samples in Fig. 1 (compare WT and mre11 graphs for total and second cell cycle incorporation in Fig.1D and 1E).

The arbitrary units plotted in the original Fig. 1D cannot be compared to those of Fig. 1E. The spotting and analysis of the ChIPs to measure BrdU incorporation in the input DNAs from the WT and *mre11Δ* cells were from two different experiments with different filters, different exposures, etc…Therefore, the signals that are quantified in Fig. 1D and Fig. 1E cannot be compared.
In the revised version, we have compared in the same filters the BrdU incorporation of PL9T163 and PL9T163 *mre11::HIS3*. We have found that BrdU incorporation is about 2 times lower in

*mre11*Δ cells compared to WT in this genetic context (not shown). Following the thinking of referee1, *mre11*Δ telomeres should contain about 4 times less BrdU than the WT telomeres (assuming a 150 bp reduction in telomere length in *mre11*Δ cells). We agree with referee 1 that we should see in theory a reduction of ChIP BrdU signals in the *mre11*Δ samples for the lagging strand telomere of about 4 fold. We see only a 2 fold reduction meaning that the RPA ChIP may contain some subtelomeric DNA or that RPA binding to telomere at the lagging strand is stronger in *mre11*Δ cells than in WT. Note that such contamination should be then also seen at in the RPA ChIP at 40 min during the fist cell cycle. Apparently, this is not the case.

6) The statement on page 6 that Cdc13p binding is slightly reduced in mre11 cells (Fig.1B) should be softened. The decrease is significant, perhaps 60-70% of WT level, and the reduction for Rpa is slightly bigger (around 50%); so, the difference of reduction between Cdc13 and Rpa1 in this background is not that dramatic as one may get impression from the author's description. In addition, the synchronization is not exactly the same between Wt and mre11 cultures and, therefore, it may not be entirely appropriate to compare directly the 45min time point for the two backgrounds.

We agree. We have clarified this point in the revised version. As we mentioned above, we have softened the point that Cdc13 or RPA binding to telomere are-affected in *mre11*Δ cells.

7) Likewise, the statement on page 7 that the presence of RPA on the leading strand telomere is at least partially dependent on Mre11 does not stand right along with the complete absence of ChIP BrdU signals in Fig. 1E.

As I mentioned earlier, the fact that we obtain no BrdU signal in the ChIP does not mean that DNA is absent from the Rfa1 ChIP but that we are below the level of BrdU to be detected by the anti-BrdU antibodies.

8) The authors should also correct their conclusion on page 9 that RPA and yKu interact with each other preferentially at the end of S-phase because it is not supported by the data. The time course experiment in Fig. 3C and 3D actually shows that RPA and yKu interact throughout the cell cycle, except in G1, including at 30min and 75min after release when RPA is not enriched at telomeres.
We agree, this conclusion has been corrected accordingly.

9) Some of the data on the RPA-yKu interaction, specifically panels 3C, 3D and 5B, could be moved to the Supplemental data section because of the above reasoning and because the reduced rfa1D228Y-yKu interaction arguably may stem from reduced binding of rfa1D228Y to telomeric DNA, not yKu (see below).

We have unpublished results showing that the binding of Rfa1 and of the rfa1D228Y mutant to resected DNA after a HO-cut are similar. Therefore, the reduced interaction with yKu does not stem from reduced binding of rfa1D228Y to telomeric ssDNA.

10) The observation that rfa1-D228Y mutant is partially defective in telomere association is rather surprising considering the crystal structure model presented in Fig. S3 and in the light of the fact that the equivalent rad11-D223Y mutation in fission yeast does not seem to affect DNA binding. Is the rfa1-D288Y mutant protein proficient in DNA binding? Or the decreased telomere association in Fig. 5A results only from defective interaction with other telomere-bound protein partners? Is the replication affected in rfa1-D288Y cells?

In their original paper, Smith and Rothstein (Mol. Cell Biol., 1995) observed that the *rfa1-D228Y* strain grew slowly, was UV sensitive, exhibited decreased levels of heteroallelic recombination, and exhibited reduced levels (two-fold) of RPA. However, these phenotypes could be totally or partially suppressed by the overexpression of the Rfa1-D228Y mutant protein. *In vitro*, they found that the Rfa1-D228Y mutant bound ssDNA as the WT protein. In ours hands, expression of Rfa1-D228Y mutant is only slightly reduced compared to WT and the *rfa1-D288Y* cells do not exhibit apparent defect of replication when cells are grown in YPD.
All these data indicate that the Rfa1-D228Y mutation impairs protein/protein interactions thereby weakening RPA telomere association.

8) The authors should carefully verify the manuscript for mislabelled figures and mistakes in figures citations in the text. Examples: in the legend of Fig.1, (C) and (D) do not correspond to the figure panels, (E) is missing in the legend but present in the figure; in first paragraph on page 15, in the brackets, 5A should be 4A... They also should take care to describe more clearly the methodology in Methods and in figure legends to facilitate understanding. For instance, in Fig.1 it is unclear the telomeres on which chromosome were analysed after ChIP (none of the 3 primer pairs listed in Methods corresponds to the chromosome specified in the figure legend) and which genomic locus was used as a control to calculate the fold enrichment (in Methods they point Gal2 but no primers shown in the Table S2; instead primers for ARO1 and ADH4 listed - why?). Also, the concentration of thymidine competitor added during the second cell cycle cannot be 2000 g/ml.

We thank referee 1 for pointing out these mistakes. They will be corrected accordingly.

Referee #2 (Remarks to the Author):
The authors previously reported that budding yeast RPA (replication protein A) associates with telomeres and regulates the telomerase reaction through recruiting Est1 to telomeres (Nat genet, 2004). In this manuscript, they propose that RPA associates with Ku proteins and that telomeric RPA recruits telomerase via the RPA-Ku and RPA-Est1 interactions. Overall, experimental data supports the authors'conclusion. However, I have technical concerns as described below.

Major concerns
1. Quantification of Chip data
I do not understand the rationale to quantify the Chip results by "the relative values of bound telomeric DNA (TelVI-R) over background" (Fig. 1, legend). The authors do not indicate what sequences they used in "background" measurements. If they measured the amount of non-telomeric DNA sequences in Cdc13-Flag or RPA-ChIP, the values are theoretically close to zero. In this case, "the relative values" should be very large and do not make sense as a measure because even small stochastic fluctuation of the background value (around zero) will produce a tremendous fluctuation of the relative value.

We are of course aware about the caveats of using relative values. We use *GAL2* or *ARO1* as non-telomeric sequences. Usually the background is not closed to zero thereby preventing small stochastic fluctuations (closed to zero) that would introduce artefactual variations. Usually, the RPA-ChIP associated background shows very little variations even by the time when the control genomic locus is replicated. The legends have been corrected accordingly.

2. The authors' conclusion that RPA physically associates with telomerase is based on a) detection of TLC1 RNA in RT-PCR experiments of Rfa1 IP; b) TLC1-RPA interactions detected by co-IP/RT-PCR showed similar changes during the cell cycle as those of TLC1-tel and RPA1-tel associations; c) TLC1-RPA interactions detected by co-IP/RT-PCR are additively defective in tlc1-delta48 and est1-delta mutation; d) TLC1 was not detected in RPA ppt in the Rfa1-D228Y background; and e) Telomere elongation in rif1-delta and/or rif2-delta was rescued the Rfa1-D228Y background.

Although all experimental data are supportive for the RPA-telomerase interaction, most are indirect evidence. Because RPA is an abundant protein in cells, authors need carefully exclude artificial associations. Authors briefly mentioned that they could not detect Est2 in RPA ppt. Did they try to detect telomerase activity coIP'ed with RPA to show that RPA associates with active telomerase holoenzyme?

As shown by our Co-IP (see further), the amount of Est2 in the RPA Co-IP is low precluding this approach. Actually, It is already difficult to show telomerase activity in Est2-IPs.

3. The authors' conclusion that RPA physically associates with Ku is based on a) reciprocal IP-IB experiments of Rfa1 and yKu80-myc; b) RPA-yKu interactions detected by co-IP showed similar changes during the cell cycle as those of yKu80-tel association; and c) TLC1-RPA interactions detected by co-IP/RT-PCR are Mre11-dependent as in the cases of TLC1-tel and RPA1-tel associations.
Fig. 2A: Co-immunoprecipitation experiments of Rfa2 in Fig. 2A are difficult to interpret. The Rfa2 bands are closely located with other intense bands (presumably derived from Ig used for IP) and the

specificity is not clear. Experiments using anti-Rfa1 should give clearer results as shown for Rfa1-Ku interactions in Fig. 3A.

Co-IP experiments shown in Fig. 2A suggest that Rfa2 is co-immunoprecipitated with Cdc13 and Est2. To answer this concern, we have immunoprecipitated Myc-tagged versions of Est2 and Cdc13 in late S-phase and analyzed the presence of Rfa1 in the Cdc13 and Est2 IPs. As shown below, Rfa1 is efficiently coimmunoprecipitated with Cdc13. Rfa1 is also weekly coimmunoprecipitated with Est2. The low amount of RPA in the Co-IP may reflect the low efficiency of the Est2 IP.
In the original version of the MS, we have shown that the association between RPA and TLC1 was resistant to the addition of DNase indicating that the interaction between Rfa1 and Est2 is not mediated by DNA. Similarly, the interaction between yKu and Rfa1 is mediated neither by DNA, nor by RNA. Moreover, we always observed that the telomere binding of RPA, Cdc13, yKu, Est1 and Est2 peaks at the same time-point. Although, based on the result of this experiment we cannot exclude that the interaction between RPA and Cdc13 is mediated by telomeric DNA, our results suggest the existence of a transient complex comprising RPA, yKu, Cdc13 and the telomerase holoenzyme.
The figure has been added to Fig. 2 as Fig. 2B.

Fig. 3A
What is the yKu80-myc signal in lane 3 of the left panel (anti-myc IP of untagged control cells)?
This is a background band that we detect sometimes in the Myc-IP and that co-migrates with the yKu80-myc. This is now indicated in the legend of Fig. 3A.

Fig. 3C
It appears to me that the migration of Rfa1 coIP'ed with yKu80-myc is slightly slower than Rf11 in the input (lower panel). If this is the case, what caused the difference?
This is gel effect.

What is the fast migrating band in yKu80-myc IP fractions (upper panel)?
This is a background band

Minor concerns
1. Fig. 1
The legends are not consistent with the actual figure, although the text refers to the correct figures. In the legends, they say (B) indicates Chip results of Cdc13-Flag and RPA, but in the actual figures they are presented in (B) and (C), respectively. In the legends, they say BrdU Chip results of wt and mre11-D cells are shown in (C) and (D), but in the figures they are shown in (D) and (E).

The legend has been corrected accordingly.

2. Page 13, "Rad3 that leads to a substantial telomere shortening (Nakamura et al, 2002; Kanoh et al. 2003)". These citations are inappropriate. The first paper showing the Rad3-deletion results in substantial telomere shortening was Naito et al. Nat. Genet. 1998.

The citation has been corrected accordingly.

Referee #3 (Remarks to the Author):

The molecular steps involved in regulating telomere elongation have focused to a large extent on understanding the interplay of telomere-bound proteins with telomerase. How more general DNA replication proteins such as RPA fit into the picture has not been entirely clear. This manuscript shows that RPA can interact with both yKu80 and the telomerase RNA, and requires Mre11 function to efficiently bind to the leading strand telomere. The model constructed from the data is that RPA binds to resected telomeres, then transiently interacts with telomerase that is present. That Mre11 function promotes RPA leading-strand telomere association is certainly consistent with RPA binding to the 3' single-strand telomere DNA following resection, as opposed to being enriched due to the replication fork moving through or pausing at telomeres. A conserved, RPA-dependent telomere

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length regulation mechanism is proposed to exist, based on evidence from both S. cerevisiae and S. pombe yeast species.
Given the conservation in the interaction between RPA and telomerase, it would be interesting to follow this data with an investigation into whether the RPA association with leading strand telomeres is affected by telomerase, or conversely whether telomerase association on the leading strand is adversely affected by loss of RPA interaction.
We agree but this represents a whole study beyond the scope of this MS.

Major points

1. The data in Figure 1 strongly indicate that Mre11 positively influences RPA recruitment to leading but not lagging strand telomeres. This is an important finding. Since RPA is not telomere specific in its DNA binding, it is curious that no BrdU was detected in the Rfa1 ChIP in the first cycle after BrdU exposure. Is the crosslinking at other genomic locations far less efficient?
See our response to referee 1.

2. Since telomeres in mre11∆ strains are short, it is important to know whether the reduced telomere length accounts for the lower enrichment of telomere fragments in the ChIP data for figure 1. It was previously reported (by some of these authors) that if one normalizes to telomere TG1-3 sequences in the analysis of Cdc13 ChIP, much of the difference in ChIP efficiency between MRE11 and mre11∆ cells goes away. However, in figure 1, TelVI-R was used to measure the telomere DNA in the ChIP. This probe will not take into account the difference in telomere length between the strains. Therefore, in the data shown, it is difficult to separate the impact of telomere length versus Mre11 absence on RPA telomere association.
We agree with this point (see our response to referee 1). As stated above, we have now softened the statement about the reduced binding of Cdc13 and Rfa1 in *mre11* cells.

3. Some conclusions the authors make in their comparisons of Cdc13 and Rfa1 telomere associations are not as well substantiated by the data as stated in the text. The data comparing Cdc13 (here and from Faure et al 2010) and Rfa1 telomere localization seems to be in fact more similar than different. As discussed further below, RPA does not necessarily seem to be more sensitive to Mre11 function than Cdc13 is. Instead, it seems that the presence of both Cdc13 and RPA on leading strand telomeres is affected by Mre11.
We agree (see above), we have amended our text accordingly.

The authors conclude from the data in figure 1 that loss of Mre11 has more of an impact on Rfa1 ChIP than on Cdc13-Flag ChIP, saying Cdc13 ChIP is only slightly reduced in mre11∆ whereas RPA ChIP is reduced by 2x. However, the data shown do not necessarily support this conclusion. From the graphs shown in 1B and 1C, it looks like the TelVI-R enrichment measurement for Cdc13 ChIP (at 45 minutes) is ~ 23/33 in mre11 / MRE11, and for Rfa1 is ~20/33 in mre11/ MRE11. The difference is more pronounced in the Rfa1 data from 1D and 1E. Cdc13 is not shown for comparison here, although such Cdc13 data was published in the Faure et al 2010 paper. Whether or not Mre11 affects RPA more than Cdc13 is really a minor point for this paper. Instead of trying to include further experiments to better substantiate a difference, it may be better to be more careful in the comments in the text.
We agree, see the previous response.

3. The cell cycle timing of Cdc13 and RPA telomere association seems to vary substantially when comparing the data in figure 1, even though the figure legend seems to suggest that these data came from the same cell cycle release experiments. Why is the peak of Cdc13-Flag and Rfa1 telomere association 45 minutes in 1B, 1C and 5A but in 1D and 1E the peak for Rfa1 it is 70 minutes? Does this mean the peak of Cdc13 and RPA telomere binding is before the region is replicated? Is the 45 minute time point really late S phase? If the legend was clearer, it may help to resolve this issue.

In the original Fig. 1B and 1C, cells are grown in YPD rich medium whereas in Fig. 1D and 1E cells are grown in SD minimal medium that contains in addition BrdU during the first cell cycle. The legend has been now clarified in the revised Fig. 1.

4. The biochemical data indicates that RPA can co-immunoprecipitate with yKu80 and TLC1. Both Rfa1 and Rfa2 were tested, and the data are strongly substantiate these associations. However, the data in Fig. 2A testing interaction of Rfa2 with Cdc13 and Est2 is not convincing. The Rfa2 band is quite difficult to see, due in part to the close migration of the antibody chain. Why not use the anti-Rfa1 antibodies for these co-IPs? Alternatively, you could avoid the IgG bands by using a chicken anti-myc antibody for the IP.

We have immunoprecipitated Myc-tagged versions of Est2 and Cdc13 in late S-phase and analyzed the presence of Rfa1 in the Cdc13 and Est2 IPs (See our response to referee 2, point 3).

Minor points

4. The figure legend (A-D) for Figure 1 does not completely correspond with the figure displayed (A-E?). A better written legend would be quite helpful.
The figure and the legend have been corrected and improved.

5. What do bars represent in ChIP figures? Error in measurement for one experiment? Standard deviation among multiple biological replicates? It is important to include this information in the paper.
Error bars represent standard deviation of at least two independent experiments. For each experiment, the RTqPCR were performed three times. This information is included in the revised version.

6. On page 7, toward the end of the first paragraph, it is written that BrdU signal was associated with Rfa1-ChIP at 50 and 70 min after the G1-releases. However, the data in Fig. 1 looks like the peak associations are at 70 min for both cycles.

The text has been corrected and adapted to the new Fig 1D and 1E.

7. On page 9, the last sentence in the middle paragraph states that RPA and yKU interact with one another preferentially at the end of S phase when both are at telomeres. This seems a bit strong, and could just be qualified a bit, as was done in the discussion. (Is the amount of Rfa1 in the yKu80-myc IP really greater at 45 minutes? yKu80 and Rfa1 are clearly seen to co-IP at 30 minutes, when RPA is not strongly crosslinked with telomere DNA. It is possible these interactions take place elsewhere in the genome or independent of DNA..)

We agree. Our statement has been amended accordingly (this point has been also brought by referee 1).

8. On page 18, in the middle paragraph, it should say that RPA stimulates the elongation of the G-strand or 3' strand by telomerase - not the 5' strand.

Thanks for pointing out this mistake. The sentence has been corrected.

1st Editorial Decision 13 January 2012

Thank you for submitting your revised manuscript for our consideration. It has now been reassessed by the three original reviewers, and I am pleased to inform you that they all consider the study substantially improved and now in principle suited for EMBO J publication. Referee 3 retains a number of interpretational and presentational concerns, which I would like to ask you to address in a final round of minor revision that will not require further experimentation. However, when revising the text there are also a few more editorial issues I need to draw your attention to:
- please include in methods and/or figure legends some brief explanations on statistical analyses of the data, where appropriate
- please make sure to remove red text markups (for changes), especially in the supplementary information as this will not be copy-edited or typeset
- finally, please take care to slightly rewrite a few passages in the introduction (1st paragraph, 1st two sentences of 2nd paragraph, 1st two sentences on page 4) and results section (2nd paragraph on

page 6), which currently appear as near-verbatim copies of passages in Faure et al 2010 and Brunori et al 2005 - simply in order to avoid possible accusations of self-plagiarism at later post-publication stages

Following these modifications and re-submission of the final revision, we should be able to swiftly proceed with acceptance and publication of the study!

Thanks and best regards,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript has been considerably strengthened by newly added data, improved methodology used for experiments in Fig.1. and straightening some of the conclusions drawn. The authors have adequately addressed most of criticism I raised on the first submission and, in my opinion, they also have satisfactorily answered the most important questions, some of which had overlaps with remarks from other reviewers as well. It should be recognized that despite RPA ChIPs, the BrdU incorporation and detection with anti-BrdU antibodies being technically challenging, the authors have done a very good job of perfecting that elegant approach. I would take the chance to suggest they employ in future a random total genomic DNA to probe the RPA immupoprecipitates instead of a unique genomic locus probe. This may help substantiate exactly what fraction of RPA is associated with telomeres in S-phase cells.
Nonetheless, the key new finding in this work that will certainly interest the telomere community is the formation of a transient complex including RPA, yKu and telomerase, which may favour the telomerase action in situations where the amount (or the time of residence) of RPA at telomeres exceeds its wild-type level, such as in rif1 rif2 cells. Thus, I trust the revised manuscript is now appropriate for publication in The EMBO Journal.

Referee #2 (Remarks to the Author):

The manuscript has been appropriately revised in response to my concerns.

Referee #3 (Remarks to the Author):

This manuscript makes some significant new contributions to understanding how RPA action at telomeres interfaces with telomerase and regulators of telomerase. The data in this revised manuscript are sound, although some correctable issues in interpretation or presentation remain, as discussed below.

Point 1. The new data for figure 1 has definitely improved in quality. A key point from Figure 1 is that Mre11 significantly impacts RPA binding to the leading strand telomere. The data are consistent with that interpretation. However, the authors need to alter their discussion of this data so that they do not suggest the BrdU signal represents only telomeric DNA. As explained further below, I don't think that the authors need to equate BrdU signal with telomeric signal in order for their conclusion to be well justified.

The conclusion that that the BrdU signal immunoprecipitating with Rfa1 in 1D and 1E represents primarily telomeric DNA is based on an assumption that Rfa1 crosslinks far more efficiently with telomeres than other genomic locations. This may be true in comparison with the unique GAL2 locus that is shown, but it may not be fair to extrapolate from GAL2 to the whole genome. Please note that the data in Figure 1E also indicate that the BrdU signal is not exclusively telomeric, as

evidenced by the cycle-2 60-minute (and 80 min) time points in the mre11-null cells. From the same ChIP sample, there is a high level of associated BrdU signal but no corresponding TG1-3 signal.

In cycle 1, the BrdU DNA is newly synthesized, and potentially less likely to be single-stranded than in cycle 2, where it would be the template for synthesis. The lower BrdU signal in cycle 1 Rfa1 ChIP as compared with cycle 2 would be consistent with this. In the absence of Mre11, even less of the newly synthesized BrdU-containing DNA could be single-stranded, not only at telomeres but also globally, potentially due in part to less resection. This is consistent with an interpretation that the authors make to explain reduced Rfa1 crosslinking to leading strand telomere BrdU DNA. I think that this interpretation is largely consistent with that of the authors, without needing to assume that the BrdU signal in the IPs is predominantly or exclusively telomeric in nature.

Point 2. There are a few places where the authors seem to suggest that they have measured how much RPA complex is associated with leading versus lagging strand telomeres. On page 7, "Therefore, in mre11 cells, RPA telomere binding at the leading strand (that accounts for 30% of the total RPA in WT cells) is strongly decreased." Then again, page 15, "Our results indicate that 70 % of the RPA that we are able to ChIP is localized at the lagging-strand telomere." These statements need to be modified since there is not data here that quantifies how much RPA is localized to telomeres, let alone with the specific strands. While the BrdU ChIP data does indicate that the crosslinking to BrdU containing DNA is more efficient in the second cell cycle, this is not a direct measure of only telomere DNA, nor of what fraction of RPA protein complex is bound to a specific site. It is certainly fair to conclude that RPA binding at the leading telomere strand is strongly decreased in mre11-null cells. However, the authors go too far beyond this. To make the statements in the paper, the authors should know what fraction of total cellular RPA is in the IPs, what percent is crosslinked to the DNA, and what percent of the crosslinked DNA is telomeric. The lack of signal from the GAL2 probe is not sufficient to conclude that the crosslinking is specific to telomeres. ChIP-seq experiments might then tell you what fraction of the total DNA in the chromatin IP was telomeric.

Point 3. Figure S5 needs to include a graph showing the percent of TG(1-3) DNA in the IP relative to the input as calculated from the dot blot. The data that is shown in S5C appears either to be from PCR (the Y axis is labeled Fold enrichment (Telo XV-L / GAL2A)), or is mislabeled. The conclusion from this data, that "RPA telomere binding increases in the rif1 rif2 double mutant (Fig. S5B) and therefore the level of telomere-bound RPA normalized to the total amount of telomeric sequences decreases by only a factor 2 (Fig. S5C)" is not written as clearly as it could be. The data presented suggest that, proportionately, there is less telomere DNA in the RPA ChIP in the rif1 rif2 mutant than in a wild-type strain. Is the comparison with Cdc13 here meant to suggest that the ratio of Cdc13 vs RPA is altered on the elongated rif1 rif2 telomeres? If so, this should be more explicitly stated, and with some analysis of whether the differences are statistically significant.

Point 4. Related to point 3 above, in the discussion, the following statement could be clarified so it doesn't suggest that this paper has measured telomere bound telomerase in different genetic contexts. Telomere length is not a direct measure of telomere bound telomerase. Page 15, "Moreover, we show by using different genetic contexts that the amount of RPA at telomeres is correlated to the amount of telomere bound telomerase."

Point 5. The least convincing data in this manuscript are the co-IP data that attempt to show Est2myc association with Rfa1 and Rfa2. (Figure 2A and 2B), but on the whole, this doesn't substantively weaken the conclusions of the paper.

Minor corrections -
"weekly" should be spelled "weakly".

Toward bottom of page 8, Cdc13-Fag should be Cdc13-Flag.

Figure 5, the "IP" labels on the left side of the panels in C should be removed. On part A, the (Telo/Back) labeling on the graphs should be corrected to be more specific.

Figure 7A - the Y axis says "Relative precipitation" and is explained in the figure legend by "bound telomeric DNA". This should be more specific as in other figures - presumably this is telomere

DNA / input rather than a fold enrichment as measured by PCR.

Page 16, "Given the fact that RPA is required for telomerase action in rif1 rif2 cells," Better to soften a bit to say "RPA promotes" rather than "RPA is required for".

2nd Revision - authors' response 23 January 2012

Thank you for your decision and for the final referee comments. We provide below a description of the changes in response to your comments and those of referee 3. We hope you will be satisfied by these final changes and wish to thank you again for your consideration.

- please include in methods and/or figure legends some brief explanations on statistical analyses of the data, where appropriate

*We have indicated in the Material and Methods section: "In all experiments, errors bars represent the standard deviations from 3 independent experiments."

- please make sure to remove red text markups (for changes), especially in the supplementary information as this will not be copy-edited or typeset

*done

- finally, please take care to slightly rewrite a few passages in the introduction (1st paragraph, 1st two sentences of 2nd paragraph, 1st two sentences on page 4) and results section (2nd paragraph on page 6), which currently appear as near-verbatim copies of passages in Faure et al 2010 and Brunori et al 2005 - simply in order to avoid possible accusations of self-plagiarism at later post-publication stages

*done

Referee #3 (Remarks to the Author):

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In cycle 1, the BrdU DNA is newly synthesized, and potentially less likely to be single-stranded than in cycle 2, where it would be the template for synthesis. The lower BrdU signal in cycle 1 Rfa1 ChIP as compared with cycle 2 would be consistent with this. In the absence of Mre11, even less of the newly synthesized BrdU-containing DNA could be single-stranded, not only at telomeres but also globally, potentially due in part to less resection. This is consistent with an interpretation that

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the authors make to explain reduced Rfa1 crosslinking to leading strand telomere BrdU DNA. I think that this interpretation is largely consistent with that of the authors, without needing to assume that the BrdU signal in the IPs is predominantly or exclusively telomeric in nature.

We agree with with Referee 1 that in *mre11*Δ cells not all the BrdU signals correspond to telomeric DNA since BrdU signals are still present at the 60 and 80 min time points of the RPA-ChIP while no telomeric DNA is associated with the ChIP (Fig. 1E). Note that this is not the case in WT (Fig. 1D). We therefore have amended our text to take into account this comment. The third paragraph of the first section was rewritten as follows:

"For each time point, we then analyzed the incorporated BrdU in the RPA-ChIP by a spot assay (Fig. 1D, E). In WT cells (PL9T163), BrdU signals were mainly obtained for the time points when RPA binds to telomeres. BrdU was detected for the two consecutive cell cycles. In contrast in PL9T163 mre11Δ cells BrdU signals were strongly reduced during the first cell cycle but not during the second cell cycle. Therefore, in mre11Δ cells, RPA telomere binding at the leading strand is decreased. Note that some BrdU was associated to the RPA ChIP at 60 and 80 min time points (Fig. 1E) suggesting that in mre11Δ cells not all the BrdU signals correspond to telomeric DNA. "

Point 2. There are a few places where the authors seem to suggest that they have measured how much RPA complex is associated with leading versus lagging strand telomeres. On page 7, "Therefore, in mre11∆ cells, RPA telomere binding at the leading strand (that accounts for 30% of the total RPA in WT cells) is strongly decreased." Then again, page 15, "Our results indicate that 70 % of the RPA that we are able to ChIP is localized at the lagging-strand telomere." These statements need to be modified since there is not data here that quantifies how much RPA is localized to telomeres, let alone with the specific strands. While the BrdU ChIP data does indicate that the crosslinking to BrdU containing DNA is more efficient in the second cell cycle, this is not a direct measure of only telomere DNA, nor of what fraction of RPA protein complex is bound to a specific site. It is certainly fair to conclude that RPA binding at the leading telomere strand is strongly decreased inmre11-null cells. However, the authors go too far beyond this. To make the statements in the paper, the authors should know what fraction of total cellular RPA is in the IPs, what percent is crosslinked to the DNA, and what percent of the crosslinked DNA is telomeric. The lack of signal from the GAL2 probe is not sufficient to conclude that the crosslinking is specific to telomeres. ChIP-seq experiments might then tell you what fraction of the total DNA in the chromatin IP was telomeric.

* We agree, we have modified these statements page 7 and 15 according to the referee comment.

Point 3. Figure S5 needs to include a graph showing the percent of TG(1-3) DNA in the IP relative to the input as calculated from the dot blot. The data that is shown in S5C appears either to be from PCR (the Y axis is labeled Fold enrichment (Telo XV-L / GAL2A)), or is mislabeled. The conclusion from this data, that "RPA telomere binding increases in the rif1∆ rif2∆ double mutant (Fig. S5B) and therefore the level of telomere-bound RPA normalized to the total amount of telomeric sequences decreases by only a factor 2 (Fig. S5C)" is not written as clearly as it could be. The data presented suggest that, proportionately, there is less telomere DNA in the RPA ChIP in the rif1 rif2 mutant than in a wild-type strain. Is the comparison with Cdc13 here meant to suggest that the ratio of Cdc13 vs RPA is altered on the elongated rif1 rif2 telomeres? If so, this should be more explicitly stated, and with some analysis of whether the differences are statistically significant.

The figure S5 was in fact mislabeled as indicated by referee 3. The Y axis in Fig. S5C and D represents TG(1-3) DNA in the IP relative to the input as calculated from the dot blot. The legend has been corrected accordingly.

The ratio of Cdc13 vs RPA is indeed altered on the elongated rif1 rif2 telomeres in favor of RPA. We did twice the experiments and obtained the same results. We may explain these results by suggesting that Cdc13 is mainly present at the end of the telomere whereas RPA is present at both, the replicating telomere and the telomere end. The text was changed accordingly:

"The results show that the total amount of Cdc13 bound to telomeres is similar in WT and rif1Δ rif2Δ cells (Fig. S5B) but when the level of telomere-bound Cdc13 is normalized to the total amount of TG_{1-3} sequences, the level of Cdc13 drops by a factor 4 (Fig. S5C). RPA telomere binding

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increases in the rif1Δ rif2Δ double mutant (Fig. S5B) but proportionately to the total TG_{1-3}*, there is less telomere DNA in the RPA ChIP in the rif1Δ rif2Δ mutant than in a wild-type strain (Fig. S5C). These results suggest that Cdc13 is mainly present at the end of the telomere whereas RPA may be present at both, the replicating telomere and the telomere end. We then measured the interaction between RPA and telomerase in the rif1Δ rif2Δ double mutant. We observed a modest increase (30%) of the association between RPA and TLC1 in rif1Δ rif2Δ cells that may reflect an increased association between RPA and telomerase"*

Point 4. Related to point 3 above, in the discussion, the following statement could be clarified so it doesn't suggest that this paper has measured telomere bound telomerase in different genetic contexts. Telomere length is not a direct measure of telomere bound telomerase. Page 15, "Moreover, we show by using different genetic contexts that the amount of RPA at telomeres is correlated to the amount of telomere bound telomerase."

We agree. We have changed the sentence at the end of the paragraph page 15 (in italics):

Using a more sensitive assay, the results presented here provide evidence for an interaction between RPA and telomerase by the time when telomerase is associated to telomeres in late S-phase. Several lines of evidence indicate that this interaction is specific. Indeed, RPA interacts with TLC1 in a narrow window in late S-phase and mutations in Rfa1 abolish the association between TLC1 and RPA. *Moreover, we show that in mre11Δ cells the decreased interaction between RPA and TLC1 is correlated to the decrease of telomere bound telomerase (Goudsouzian et al., 2006).*

Point 5. The least convincing data in this manuscript are the co-IP data that attempt to show Est2myc association with Rfa1 and Rfa2. (Figure 2A and 2B), but on the whole, this doesn't substantively weaken the conclusions of the paper.

Minor corrections -

"weekly" should be spelled "weakly".
*done

Toward bottom of page 8, Cdc13-Fag should be Cdc13-Flag.
*done

Figure 5, the "IP" labels on the left side of the panels in C should be removed. On part A, the (Telo/ Back) labeling on the graphs should be corrected to be more specific.
*done

Figure 7A - the Y axis says "Relative precipitation" and is explained in the figure legend by "bound telomeric DNA". This should be more specific as in other figures - presumably this is telomere DNA / input rather than a fold enrichment as measured by PCR.

*done

Page 16, "Given the fact that RPA is required for telomerase action in rif1∆ rif2∆ cells," Better to soften a bit to say "RPA promotes" rather than "RPA is required for".

*done