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Telomere capping in non-dividing yeast cells requires Yku and Rap1

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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, and please excuse the delay in getting back to you with a decision, related to the high number of submissions and the editorial office closure around the turn of the years. We have now received the reports of three expert referees, which you will find copied below. While these referees seem to differ considerably in their overall opinions and recommendations, it is at the same time apparent that many of their main criticisms represent in fact shared or overlapping concerns. As it stands, we feel that the extent and importance of these criticisms precludes publication of the study, at least in its current form. Given that they however pertain mostly to technical and experimental issues that you may well be able to address, I would nevertheless be open to consider a revised version of the manuscript if you should feel confident that you might be able to satisfactorily respond to the most salient points raised by the reviewers - including the key criticism whether a described G1 telomere attrition defect (Fig. 2A) may be considered significant or not. Overall, it appears clear that adequately addressing the main concerns would likely require a significant amount of further time and effort and also may not necessarily result in the substantiation of the conclusions asked for by the referees, so I would also understand if you were rather to decide to rapidly publish the manuscript without major changes elsewhere in the meantime. Should you decide to revise the manuscript for The EMBO Journal, I would like to remind you that it is our policy to allow only one round of major revision, and that it will thus be important to diligently and thoroughly answer to the various comments at this stage of the process. In this respect, please also carefully look into the question of composite images in panels 2B, 3A (as raised by referee 1) and also for all other data, as this issue is currently somewhat difficult to discern due to the limited resolution of the provided PDF figure file, but nevertheless a key issue for publication in any major journal (please read on below and in our author's guidelines for our policies on digital figure preparation and assembly).

Finally, when preparing a 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):

This manuscript shows that in G1 arrested cells, the essential telomere capping protein Ten1 become dispensable for cell viability, supporting the previous finding by the same group that non-dividing cells do not require the other two protein in the same complex, Cdc13 and Stn1. The authors also went on to establish that Yku and Rap1 are involved in capping telomeres in non-dividing cells. Overall, the findings that Yku and Rap1 caps telomeres in non-dividing cells (and dividing cells) is potentially interesting and the data supporting it is generally consistent with the hypothesis. However the quality of the manuscript is seriously affected by poor data that does not support several arguments laid out by the authors. Furthermore the manuscript did not seem particularly coherent with the Rap1 results not really following on from the other experiments.

Overall I do not support publication of the current manuscript. There are some interesting observations but I think they need much more "work up" before being appropriate for EmboJ.

Major comments:

General criticism) Much use is made of degron alleles. As far as I understand each degron strain (protein) behaves differently i.e. At one end of the spectrum about 30% of proteins cannot be degraded at all (copper, UBR1 overexperession, 37oC), but at the other end some proteins are completely unstable under all conditions. Therefore each degron mutation must be very carefully characterised to determine its phenotype, to deterine where on the spectrum of instability it lies.

1) In Western blots to show degradation of degron proteins (Fig. 1B, Fig. 5A and Fig.S3A), loading controls are required to support the statements that the protein have been degraded

2) The authors used Fig. 2A to show degradation of telomeric DNA in yku-td mutants in G1-arrested cells, however, the data does not look convincing. The authors said that this telomere resection occurred at a comparable level in a yku-td cdc13-td double mutant strain, but Fig 2A clearly shows that yku-td cdc13-td strain has more ssDNA than a yku-td strain. the yku70td result in G1 is far from striking!, the adjective the authors used

3) The author concluded from Fig. 2C that ku70-30ts mutants have more telomere degradation, but this is not convincing as the loading control is not equal. Also it is better to test the effect of Yku in ku70-30ts single mutant instead of combining it with cdc13-td because cdc13-td mutation could have subtle effect on telomere even in Raff. The same applies to Fig. 3A

4) In Fig. 3B, the authors said that there is no Rad53 phosphorylation in yku-td cycling cells, but there is clearly a shift in Rad53 mobility (lane 8 and 9). Generally this data is poor quality.

5) From Fig. 4A/B, the authors suggested that MRE11 deletion suppressed ssG-tail accumulation

after Yku70-td depletion in G1-arrected cells, but Fig.4B clearly showed that this is not the case. The authors also suggested that Mre11 and Exo1 co-operate in the processing of uncapped telomeric ends, but there is no data to support this statement.

6) Fig. 2B, 3A appeared to be composite images it should be made clear if this is so, and if for example the spot tests were done on the same plate or at the same time.

7) What is the idea behind the very unusual time for treating rap1-td strains in gal. Why 16h, followed by dilution and 3h? This seesm very strange to me.

Minor comments:
1) Generally the English is poor and there are numerous typos and grammar mistakes throughout the manuscript.

2) It also does not help make constructive criticisms when there are no page numbers on the manuscript..

Referee #2 (Remarks to the Author):

This manuscript examines which proteins protect telomeres from degradation in non dividing cells. While the CST complex appears to be dispensable for telomere protection in the G1 phase of the cell cycle, inactivation of Yku70 in G1 cells leads to moderate telomere degradation. Furthermore, the Rap1 protein is important to prevent ssDNA generation at telomeres in quiescent G0 cells. Telomeric ssDNA generation in G1-arrested cells lacking Yku appears to involve both MRX and Exo1 nucleases, whereas Exo1 is required only in cycling cells for ssDNA formation after Rap1 loss.

I think this work is interesting. However, several additional experiments are required to unequivocally support the authors' conclusions, as detailed in the specific critique points.

Specific critique points:

1. The authors have analyzed the effect on telomere protection of Yku70 inactivation in -factor arrested cells (and therefore in G1) and in quiescent cells (a condition of prolonged stationary phase, which they call G0), while the effects of Rap1 inactivation have been assessed only in quiescent cells (G0), due to the impossibility to arrest Rap1 lacking cells with -factor. They use G0 to recapitulate G1, and so to strongly suggest that Rap1 is involved in telomere protection in G1 (see running title, for example). I think this assumption is not appropriate, as G1 and G0 are two completely different conditions, at least from a metabolic point of view. Their results simply indicate that Rap1 protects telomeres in G0, while Yku does it in G1 and G0, but there is no clear connection between the two conditions and the different nuclease requirements for Yku in G1 and Rap1 in G0 might be related to the different cell cycle conditions. In my opinion, it would be better to focus the manuscript on G0, and show the role of both Yku and Rap1 only in quiescent cells.
2. In order to demonstrate that Rap1 depleted cells are in G0, the authors show only the FACS profiles 16 hours after shift in galactose at 37{degree sign}C. Usually, the first nutrient that becomes limiting in saturated cell cultures is the carbon source, and the addition of galactose (another carbon source) to stationary wt and rap1-td cells should induce a re-entry into the cell cycle that can stop at later time points if nitrogen becomes limiting. In order to exclude that galactose addition induces re-entry into the cell cycle, and therefore that cells are really in G0 during Rap1 depletion, the authors should show the FACS profile (or the budding profile) at different time points after galactose addition.
3. Deletion of RAP1 is lethal, most likely because of Rap1 role in transcriptional activation of some essential genes. Furthermore, mutations in RAP1 have a variety of consequences that include loss of silencing at the HM loci and at telomeres, telomere fusions, and chromosome loss. Thus, I find it unfortunate that all the work on Rap1 was done using the degron Rap1-td allele, which compromises all Rap1 functions. I believe the authors should verify that the observed telomere deprotecting effects of Rap1-td inactivation are directly caused by Rap1 loss and not by secondary effects of the latter. Specific rap1 mutants, where only Rap1 telomeric function is compromised, are available and should be used to measure telomeric ssDNA generation at least in cycling cells.

4. Based on the finding that Yku70 lacking cells (yku70-td) do not activate the checkpoint in G1, the authors conclude that telomere resection is confined to the telomere tips and that Rap1 could limit ssDNA generation in these cells. However, as the system used to detect ssDNA generation does not allow to determine the extent of degradation, the authors should test directly the possibility that yku70-td telomere resection is confined to the tips. Furthermore, also Rap1 lacking cells (rap1-td) fail to activate the checkpoint, although their amount of telomeric ssDNA seems to be higher than that of yku70-td cells. So, which are the differences between yku70-td and rap1-td cells in term of ssDNA generation?
5. I am rather confused about the nucleases involved in telomere resection upon Yku or Rap1 inactivation. The data show that both Exo1 and MRX are required to generate ssDNA in G1-arrested yku70-td cells, whereas Exo1 seems to be required for telomere resection in rap1-td cells only when they are cycling, and not when they are in G0. So, which nuclease(s) is doing the job in quiescient rap1-td cells? Is MRX required for telomere resection in these cells? Are Exo1 and MRX required to generate ssDNA also in G0 yku70-td cells?
6. Given the standard deviations shown in figure 4B, the differences between yku70-td and yku70-td sae2 cells are overinterpreted.

Referee #3 (Remarks to the Author):

In their manuscript "Telomere capping in non-dividing cells requires yKu and Rap1", Vodenicharov and Wellinger genetically dissect the role of the CST complex, yKu and Rap1 in telomere end-protection. Numerous studies implicate the CST complex as a main player in preventing runaway telomere 5' resection in cycling cells. This study sheds light on two long-known telomere players (yKu and Rap1) as well as CST in preventing resection during the G1 phase of the cell cycle. Surprisingly, while the CST complex is dispensable for telomere protection in G1 phase (as shown previously by this group), telomeres lacking yKu suffer an Exo1-dependent resection in G1. Rap1 depletion leads to substantial Exo1 dependent resection both in cycling and G1 arrested cells. Notably, neither yKu nor Rap1 loss triggers checkpoint activation.
These data argue for a functional flexibility of telomeric components at various stages of the cell cycle. These findings will greatly interest the EMBO readership.
Nevertheless, several issues need to be addressed to strengthen the clarity of the drawn conclusions:

1. The whole manuscript relies on a single method (pioneered by Wellinger and mastered by his group): in-gel hybridization. It is nonetheless difficult to understand how the authors defined the dividing line between the presence and the absence of signal. For instance, the authors state that telomere resection occurs at comparable levels in G1 cells lacking yku70 or both yku70 and cdc13, but to the untrained eye, figure 2A suggests that the yku70-td cdc13-td double experiences enhanced G1 resection compared to yku70-td alone. Quantitation (as in Fig 4B) would be useful in this regard and ideally in all figures.
2. To assess the level of single-strandedness, the denatured signal is used as reference (loading control). Most of the gels only show a narrow window of the denatured gel. Even if the digestion used does not allow a clear resolution of telomeric fragments, a "bigger picture" of what happens will be appreciated. For instance, is it primarily the longer or shorter telomeres (as assessed on the denaturing gels) that have the greatest overhang signal? In the rap1-td, does the greater signal at the lower molecular weight range reflect greater signal on the denaturing as well? The authors do not even tell us which segment of the gel has been excised in the shown denaturing panel.
3. Fig1B, the authors should comment on the very low abundance of Cdc13 compared to Yku and Ten1. Presumably Cdc13 is that much lower in abundance but does the degron-tag alter the overall protein stability even in non-inducing conditions?
4. From Fig2C, the authors claim that a ts mutant of yKu (ku70-30ts) phenocopies the effects of yKu-td. However, the ts appears to confer a much weaker single strand signal than vector alone, especially when compared to the denatured panel below. Again quantitation would help to clarify.
5. The td-tag allows protein degradation in 2h time. In the case of yKu, the overhang signal is analyzed after 4h (Fig1A) and the checkpoint (rad53 phosphorylation, Fig3B) after 5h. It has been recently shown that various players are involved in resection at DSBs with various kinetics (Exo1 participating in a very fast process). The authors should comment on differences in the timing of protein degradation, G-tail appearance and checkpoint activation.
6. S. pombe cells harboring uncapped telomeres (taz1D) do not activate a checkpoint despite having enhanced overhang signals. Resection in this case requires Dna2 but not ExoI (Tomita et al, MCB

2003 ; Tomita et al MCB 2004) - these differences with budding yeast should be cited and discussed. It might also be compared that the taz1D overhang signals persist in G1 (Ferreira & Cooper, GD 2004) but the effect of acutely inactivating Taz1 in G1 hasn't been tested as it has here for Rap1, yKu and Cdc13.
7. The authors assimilate cerevisiae Rap1 as an ortholog of pombe Taz1 and human TRF1/2 (in the Results section under 'Rap1 stabilizes telomeres..'. The direct telomeric DNA binding of scRap1 might mimic Taz1 or TRF1/2 functions but this statement should be clarified, as scRap1 is homologous to pombe and human Rap1, but not Taz1 or TRF2.
8. The Intro mentions that NHEJ occurs 'after complete removal of G-tails' but this hasn't been shown in either paper cited or perhaps anywhere - neither Deng et al, 2009 nor Zhu et al, 2003 directly shows that G-tail removal by Mre11 or ERCC1 is the basis for their role in NHEJ. E.g. since TRF2 inhibition only results in G-tail signal disappearance in a Lig4-positive cell (ie there's no G-tail removal per se - rather Lig4-mediated fusion causes disappearance of the G-tail), the ERCC1/XPF effect could reflect NHEJ causing G-tail removal rather than vice versa.

1st Revision - authors' response 17 May 2010

Concerns by Editor:

Make sure that the quantifications used support the notion that results are significant (Fig. 2A).

As also mentioned below for Rev. 1, this specific experiment was now repeated a number of times and overhang signals were quantified as indicated in the revised materials and methods. On the new Fig. 2A, we thus now include corrected mean values for ssG-tail signals and the statistical analyses of those data show that the signal for the yku70-td strain in G1 is significantly different from wt or cdc13-td ($p<0.002$, see Fig. 2 legend).

Make sure that composite images are reported (2B, 3A) and that for all composite images, full uncropped images are supplied.

As mentioned for rev. 1, Fig. 3A is not a composite and 2B is now indicated as such. Further, we do supply full plate image in Supplemental Figure 8.

Re-verify all figures for composites and indicate with blacks, if the image is not contiguous. Supply full figures of such cropped images.

In the revised version of the manuscript, we have added black bars to all such instances (sorry for our previous missing of fig. 5), and now do indicate in the Materials section that all non-contiguous images are available as full images in the Suppl. Fig. S8.

Concerns of Reviewer 1)

This reviewer mentioned that there are interesting observations reported in the paper, but that there is a need for further workup before publication.

1) Each degron allele must be carefully assessed for degradation sensitivity.

We completely agree and therefore thoroughly analyzed expected phenotypes (i.e. viability, ssG-tails etc, see Figs. 1A, 2B and S3; see also Vodenicharov and Wellinger, Mol. Cell 2006) as well as the physical disappearance of the proteins of all our alleles by westerns. The westerns mentioned by the reviewer now have been reprobed with an antibody against Sir2p for loading control and those data are now shown in separate panels of Figs. 1A (formerly 1B), 5A and S3A, as requested. We also wish to emphasize that this very same concern did push us to repeat all the pertinent experiments done with the yku70-td allele with the yku70-30ts allele. If the used td allele in some way would affect the experiments in an allele-specific fashion, we would expect a different loss-of-

function allele not to yield the same results. However, all results obtained with the yku70-30ts allele paralleled those of the yku70-td allele in a remarkable fashion (see also below pt 3).

2) The telomere resection in G1-arrested cells carrying yku70-td allele (Fig. 2A) is not convincing.

This specific experiment was now repeated a number of times and overhang signals were quantified as indicated in the revised materials and methods. On the new Fig. 2A, we thus now include corrected mean values for ssG-tail signals and the statistical analyses of those data show that the signal for the yku70-td strain in G1 is significantly different from wt or cdc13-td ($p<0.002$, see Fig. 2 legend).

3) Similarly to pt 2, the increase in resection in the yku70-30ts allele is not convincing (former Fig. 2C and 3A). It would be better to analyze the yku70-30ts allele alone and not in combination with cdc13-td.

We thank the reviewer for pointing this out to us. All these experiments have been performed now (new Supp. Figs S1, S2 and Fig. 3A). Furthermore and as was done for the gels for the yku70-td allele above, the signals in the gels were quantified and relative values now are indicated for each sample (Supp. Fig. S1A). The data confirm all of our initial conclusions for the yku70-30ts allele and completely parallel the findings made with the yku70-td allele. We hope that the reviewer now can concur that this quantification of the data plus the fact that the same results were obtained via independent loss-of-function alleles render the conclusions convincing.

4) The Rad53 phosphorylation western shown in Fig. 3B is of poor quality.

This particular experiment has been rerun and we now show a different Western on Fig. 3B bottom. We believe that the looks of the bands now is more in line with what other publications show. Furthermore, the overall conclusions taken from this experiment remain the same. In this repeat experiment, we cannot discern any anomaly with the bands in lanes 8 and 9.

5) The data in Fig. 4A/B is inconclusive and do not allow the conclusions drawn.

We agree that the quantification of these data in the original paper perhaps did not really allow strong statements. However, we have repeated those experiments again and in this new version of the figure, more data were included resulting in much more reliable data with smaller standard deviations. We now also include a statistical analysis of these data and report the corresponding P-values for the most important combination of values to compare (see new figure legend, Fig. 4). Again, we agree that these additions render the results more convincing and clear.

6) Are figures 2B and 3A composites and if yes, mark that accordingly.

All spot tests in Figure 3A are images of the same plate (not composites). The spot test in Fig. 2B is a composite, but all rows were grown on plates with all controls. In order to save space for the final figure, irrelevant lanes also present on plates were removed. We indicate that in the new figure legend now. Please note that we now also show the full plates used for the composite images in Suppl. Fig. 8.

7) The treatment of the rap1-td cultures for the inactivation experiments in G0 is strange.

We apologize for not being clear on this point. As we now try to better explain in a new section in Supplemental Methods, the rap1-td loss in G0 saturated cultures takes much more time than in cycling cells. We do not know the precise reason for this, but it could be related to the amount of Rap1 or some other difference in the degradation efficiency in these cells. However, after 16 hrs, there is no more Rap1 visible on western blots. For these reasons, we used a time of 16 hrs of induction (see blot in Fig. 5A). We also noted that after this length of incubation, even wt cells did not respond to Phleomycin (data not shown). However, after re-dilution they do (see Fig. 6C, lane 4) and so do the rap1-td cells (Fig. 6C, lane 8). Thus, despite the ability to respond to DNA damage, the rap1-td allele containing cells do not show detectable Rad53 phosphorylation after loss of Rap1,

which is our conclusion. Of note and as already mentioned above, this is true for both the undiluted and diluted rap1-td cultures (see Supplemental Methods, page 2).

8) Minor comments: Poor English and numerous typos and grammatical errors.

Again, we apologize for the inconvenience. We now had the final version re-read by a native English speaker and hope it is better.

9) Minor comments: insert page numbers.

Page numbers have been added.

Concerns of Reviewer 2)

This reviewer also thought that the work was interesting. However, some additional experiments are needed to support some of the conclusions.

1) Experiments were done in G1 and G0 (stationary phase), but these two states are metabolically different. Nuclease requirements may differ between the two states for example and the paper should focus on G0 cells only.

We do share this reviewer's opinion that G1 and G0 are different states. Although we would have liked to focus the investigations on G1 cells, the unfortunate inability of arrest the rap1-td cells with alpha factor forced us to use an alternative for those cells. In order to investigate the G0 capping in more detail, we did perform the nuclease requirement experiment as done in Fig. 4 with G1 cells now with cells arrested in G0, as suggested by the reviewer (new Supp Fig. S4). As can be observed from the quantification of these new data, the nuclease/yku70-td double mutants tested here all behave the same, whether the cells were arrested in G1 or in G0. This is also true for cdc13-td cells (Fig. 5C). Thus, while we cannot exclude that these two states differ in terms of telomere capping mechanisms, we have yet to encounter a situation where we do obtain differing results. We hope to make that more clear in the revised writing of the paper. Nevertheless, investigating telomere capping in detail in G0 cells does seem a great idea and perhaps we will investigate that in the future.

2) Show a time-course of FACS analyses of rap1-td cells after galactose addition to document that the cells remain arrested.

A very good suggestion for this control. This analysis had been carried out when we did the experiments and we now do show these data in the new Supp. Fig. S5. The profiles show that there is no discernible entry into S-phase, as stated in the paper.

3) In addition to the rap1-td allele, use specific mutations that only affect the telomeric functions of Rap1 to analyze the capping function.

While we totally agree that using such telomere specific alleles for our analyses would be a great addition, we are unsure whether such clean separation-of-function mutants actually exist. They would have to display loss of all telomeric functions, which by definition would have to include loss of DNA-binding, while keeping transcriptional regulation function, which also depends on DNA-binding. Nevertheless, we obtained certain ts and other alleles of RAP1 and carried out the experiments as requested by the reviewer. The results show a loss of capping and a slight increase of ssG-tails in some of the mutants analyzed, which supports the data with the rap1-td allele. However, the ssG-tail signals in these hypomorphic mutant strains clearly do not reach the levels observed in the rap1-td strain and none of our conclusions depend on those results. These new data are now shown in Supp. Fig. S7.

4) Measure the extent of DNA resection by other means than just in-gel hybridization for both the yku70-td as well as the rap1-td alleles.

This suggestion indeed is a very good one and we chose to use two other approaches to assess the

extent of resection (new data in Fig. 6E and F). The first is a more sensitive variation of the in-gel procedure and the second probes deep resection into subtelomeric regions by hybridization. Both techniques show deep resection into subtelomeric regions for the cdc13-td allele, which thus serves as positive control. However, neither technique revealed significant resection into subtelomeric regions for the yku70-td or the rap1-td alleles, thus confirming our conclusion that resection is limited to the telomeric repeat area for both. The increased signal in rap1-td could thus be explained by slightly deeper resection in the repeats themselves. These new results thus reinforce our initial conclusions and are valuable additions to the paper.

5) The nuclease requirements are not clear; add data on nuclease requirements for yku70-td and rap1-td cells in G0.

It is indeed intriguing and interesting that the nuclease requirements for the different mutants in the different cell cycle stages are so divergent. We believe that this in fact is one of the interesting parts of our manuscript. It will take us some time yet to figure this all out as it will require a lot more experimentation for settling all issues associated with this question. Nevertheless, as suggested by the reviewer, we did assess resection during G0 in the yku70-td/nuclease double mutants (see new supp Fig. S4). The results are virtually identical to those obtained for cells in G1 and the answer to the reviewr's specific question is yes, both MRX and Exo1 are required for resection in G0 (see Supp. Fig. S4).

6) The Sae2 data shown in Fig. 4 are overinterpreted.

As mentioned above (pt 5, rev 1), we reran many of those gels shown in Fig. 4 which now allowed us a much more tight quantitative and statistical analysis. These results and the P-values attached to the interpretations are now indicated in the new legend of Fig. 4.

Concerns of Reviewer 3

This reviewer feels that our results will be of great interest to the readers of EMBO J. Some additional results however are needed to strengthen the conclusions.

1*) Add quantification to the in-gel analyses and ideally use other techniques as well. This will allow more conclusive statements.*

This point was also raised by the other reviewers and as mentioned above, we have redone many gels, added relative signal quantifications to the relevant gels and also performed statistical analyses (P-values, see legends of new figures 2, 4 and S1). Furthermore, in order to assess the depth of resection in the yku70-td and rap1-td strains, we now also used some other techniques that do show if resection reaches subtelomeric areas, as is the case in cdc13-td strains (see the new data in Fig. 6E and F). We thank the reviewer for these suggestions as we concur that they do strengthen the conclusions.

2) Which part of the gels is used as loading control in the denatured conditions? Is there a possibility to assess whether the longer or shorter telomeres have more overhang signals? Is there more signal in the lower part of the rap1-td gels?

We apologize for this oversight. In the revised Materials and Methods section, we now spell out exactly what window was used to show for the denatured gel (see page 17 under the heading In-gel hybridization analyses). Thus, we do show the lower and important part of the gels that corresponds to the TRFs in the denatured windows shown and clearly, there is not more signal associated with the TRFs in the rap1-td strains (see Fig. 5C, 6A, B) Unfortunately, space constraints in terms of figure spaces prohibit us from showing all complete gels, as would be preferred. However, in the supplemental figures, these constraints are less of a concern and we do show complete native and denatured gels in Supplemental Figures S6 A, B and C, as well as Supplemental Figure S7.

3) Comment on lower abundance of Cdc13 versus Ten1 suggested in Fig. 1B (now 1A) or does the tag lower the stability of cdc13-td protein at permissive conditions?

We acknowledge that the western signal intensities in this Figure look as if the cdc13-td protein has a lower abundance than ten1-td or yku70-td proteins. However, we are not convinced that this is indeed the case as the individual fusion proteins may react in a differential fashion with the antibody. Further, cdc13-td is a very large protein that may not transfer very well to the membrane and there are other complications that render such an interpretation difficult. Furthermore, as we have shown previously, the cdc13-td allele does not alter telomere homeostasis in any detectable way when cells are grown in permissive conditions. Thus, if there is a reduction in cdc13-td amount in permissive conditions, it is not to a point where any phenotype could be detected (Vodenicharov and Wellinger, Mol. Cell 2006). The important point from the blot shown here is that all proteins are reduced to undetectable levels after the degron induction.

4) Quantify the single-strand DNA signals for the various yku70 alleles in G1 for comparison purposes.

As mentioned above and as also requested by the other reviewers, these quantifications have been performed and added to a number of gels, including those the this reviewer mentions in this comment (see new Fig. 2, 4, S1 and their respective legends for the statistical analyses).

5) Comment on the differences in timing for protein degradation, ssG-tail analysis and Rad53 phosphorylation.

We do acknowledge that we did not use the precise same timing for the analyses of those various parameters. In fact, our experiments here were oriented by being most certain that the tdñtagged proteins had indeed been degraded to undetectable levels. Furthermore, we did not intend to make any kinetic arguments that would require such tightly timed and coordinated analyses covering a certain time period (time course experiments with all analyses). Nevertheless, we completely agree that this will be of high interest and future research in our lab may actually probe these mechanistic details with new experimentation. However, the focus of this first paper is not on resection kinetics but setting the ground rules for which factors have to be dealt with when studying this problem. Thus, we believe those kinetic studies would go far beyond the scope of this paper here.

6) Taz1-lacking fission yeast cells also do not activate checkpoints and they do display ssG-tails in G1, as observed here. These findings need to be incorporated in the discussion.

Indeed, our initial discussion may have lacked some depth in the treatment of the fission yeast results. In the revised discussion we incorporated the most important points of these results (see end of discussion, page 16), even if, because of space constraints, a detailed discussion was not possible.

7) Budding yeast Rap1 has homologous proteins in humans and fission yeast, but it is not an orthologue of Taz1 and TRF1/2.

We agree that this passage in the original text was confusing and it was revised to say that "Telomeric dsDNA binding proteins in fission yeast and mammalsÖ." and lacking a reference to homology or orthology (see bottom of page 9).

8) For mammalian cells, the temporal relationship between ssG-tail removal and NHEJ is not demonstrated as suggested in the introduction.

We agree with this seemingly subtle but important point and have revised the introduction accordingly (see middle paragraph, page 3).

Other points that were added:

While performing the experiments requested above, we also managed to create a yku70 /rap1-td double mutant with which we were able to investigate their epistatic behaviour in terms of capping function. These new results are now reported in Fig. 6B and suggest that these two pathways affect capping differently, which is a little unexpected and exciting.

2nd Editorial Decision 16 June 2010

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the original referees 1 and 3. Referee 3 considers the manuscript significantly improved in response to the original reports, but referee 1 still retains a number of major reservations regarding the experimental support for the conclusions. To ensure a fair editorial evaluation of your study (also in light of the results recently reported by Bonetti et al), I additionally discussed the remaining criticisms of referee 1 with referee 3, who agreed that while some of these points were well taken, some of the other requests (especially points 3 & 5) may appear less important in light of the overall evidence presented. In light of referee 3's additional input, I have thus decided that we should be able to proceed with publication of your paper if you should be able to adequately respond to points 1, 2 and 4 of reviewer 1, to explain the surprising intensity variations on the denatured gels, which would appear relevant to the conclusion that MRX is required for resection (except for quantification of the shown dot blots, this may not necessarily require new experimentation).

I am therefore returning the manuscript to you once more for a last round of modification to deal with these remaining issues. When sending us your final version, please nevertheless also briefly comment on all the other points in your response letter. Furthermore, I noted that Supplementary Figure S6 may still require the addition of separation bars between grouped lanes on a gel - please check.

I am hoping you will be able to send us a modified manuscript and response letter as soon as possible, so that we could swiftly proceed with eventual acceptance and publication in this case. Please let me know if you should have any further questions in this regard.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This revised manuscript shows that in G1 arrested cells, the essential telomere capping protein Ten1 become dispensable for cell viability, supporting the previous finding by the same group that non-dividing cells do not require the other two proteins in the same complex, Cdc13 and Stn1. The authors went on to establish that Yku and Rap1 are involved in capping telomeres in non-dividing cells. Overall, the finding that Yku and Rap1 cap telomeres in non-dividing cells (and dividing cells) is interesting and the new data added make most of the results believable. Furthermore, another paper that came out this week has reported the similar finding that Yku and Rap1 are required for telomere protection in G1 (Bonetti et.al PLoS Genetics 2010), thus supporting the conclusions of this paper.

The authors have addressed most of the issues raised by the reviewers in the first version. However, there are still some experimentally weak figures that affect the overall quality of the paper and make it difficult to support publication of this paper as it stands.

Major outstanding issues are listed below.

1. Figure 4A- from figure S8G, it seems from the denaturing gels (S8G) that two very different gels presented in Fig 4A are presented more or less in a single panel, yet to this reviewers eyes they have very different loading (compare the high molecular weight bands, on the right versus left in the bottom panels of Fig S8G. Thus making it hard to compare between lanes from two different gels. This seriously affects the conclusion that resection in G1-arrested yku70td is dependent on Mre11. I

think that at the minimum I would have expected the authors to run the relevant strains on the same gel at the same time, and this would have also removed the need to show "irrelevant" lanes in the Supp data.

2. Figure 5C- from figure S8I, again it seems from the denaturing gel that the two gels presented have very different loading (compare the high molecular weight bands), thus making it hard to compare the lanes from two different gels. This seriously affects the conclusion that there is increased resection in cycling radp1-td cells. Figure 5A provides better support for this statement though.

3. Figure 6B- the small difference between telomere degradation in rap1-td and rap1-td yku70 does not support the conclusion that Rap1 and Yku function in separate pathways in G0 telomere protection.

4. Figure 6F and associated discussion. Quantification of signals and rigorous, mathematical statistical approaches are required to say whether or not something is "significant". From the figure 6F, it seems that following degron degradation, yku70-td strain shows stronger hybridization signals under native conditions, suggesting that there is also some Y' sequence resection in yku70-td strain, albeit less than in the cdc13-td strain. This data is consistent with what has been previously reported by others.

5. Many experiments with yku70-td, cdc13-td, ten1-td mutants were performed in G1 with no data confirming that the degron proteins have been degraded in G1 cells. It is very possible that degron proteins are degraded in cycling cells like in Fig. 1A, but not in G1 cells. Furthermore in Fig 1A the stability of cdc13-td could very well be affected the degradation, or mutation, of ten1-td, or vice versa. The Western blots should therefore be performed when degron alleles are present on their own and under the relevant experimental conditions (G1, G0, galactose etc.)

Minor issues:

Typos:
1. A direct role for Yku in maintaining stable telomeres is G1 (pg6)

2. ku80-135i should be yku80-135i (pg7)

2nd Revision - authors' response 18 June 2010

In the decision letter of the editor, we were requested to comment on pts 1, 2 and 4. However, we will address all points still unclear or seemingly requiring adjustments.

Point by Point answers to the remaining concerns of reviewer 1 :

Points 1. and 2. in fact are on the same issue: Our quantifications of signals for single-stranded DNA were made in various gels and from the supplied materials, it would appear that some gels had different DNA loadings. The reviewer questions whether this would have an influence on our conclusions on the MRX-epistasis experiment (Fig. 4, complete gels in SFig8G).

We hoped that our descriptions of the way these signals were quantified were clear (see Materials and Methods, p. 17-18). Signals for ssDNA always are quantified on the native gels and corrected for DNA loading, lane per lane, by re-quantification of signals obtained from the very same gel after denaturation. Then, in order to be able to make gel to gel and experiment to experiment comparisons, DNA derived from a control wt strain present on each gel was taken as the relative standard (see 3rd and 4th line, page 18). Thus, whether or not there is the exact same DNA loading in all lanes and gels becomes irrelevant and we now highlight that with a new sentence added in this paragraph: "This procedure ensures that the obtained relative signals can be directly compared between lanes, gels and independent experiments, irrespective of DNA loading differences." (this new text is highlighted in the revised manuscript). This way of quantifying the signals thus allows us

to compare very different gels from different and biological independent experiments, which according to us is the statistically good way of getting statistically relevant data with meaningful error-bars as required in Fig. 4.
The impression that DNA loading was so different in the gels mentioned (taken from SFig. 8G and SFig 8I) derives from the fact that for some gels, we did make film exposures for the denatured gel (right of SBG) but in some cases we just exposed to the phosphorimager (left on S8G). Thus, the actual and unaltered images shown in S8G appear very different, but in reality have quite comparable amounts of DNA. Note also that the native gels (upper two gels in S8G) both are images of films and thus very comparable. Again however, for the actual quantifications, these impressions do not matter and the comparisons made are, in our opinion, fully valid. The same arguments obviously also apply to pt 2 with Fig. 5C and S8I.

Point 3. A relatively small difference in signal between the rap1-td and the rap-1td/yku70td strain does not support the conclusion of a different genetic pathway.

We actually agree with the reviewer on this point: as a standalone experiment, these data would not be sufficient to derive this conclusion. Therefore, in the original text to this experiment we actually say that this difference is :" Ö. consistent with the notion that Rap1 and Yku function in separate pathways in G0 telomere protection." (sentence unchanged highlighted on page 10). We believe that this statement is adequate. At any rate, the conclusion mentioned by the reviewer is based on the behavior of other double-mutant strains described in the paragraph before the one described here on page 10.

Point 4A. To call data significant, one has to have statistics for it.

The reviewer refers to our use of "significant" for a signal observed on a slot blot (Fig. 6F; text on page 11). We felt it was rather clear that the cdc13-td strain was the only one amongst the strains tested yielding any signal in this experiment and used significant to describe that. In light of the fact that this use of significant formally may be incorrect, we have revised the sentence to "...only the cdc13-td samples yielded readily detectable hybridization signals." (see the new text on page 11).

Point 4B. The reviewer thinks that there may be a difference in the two lanes pertaining to the yuk70-td DNA (more signals after degron induction, i.e. after Yku-loss, which would be consistent with data from the Lydall lab).

The difference referred to here is very, very weak. Such differences also occur at comparable intensities in lane to lane comparisons when the experiments are repeated. Thus, we do not comment on them and we prefer to leave it that way. Perhaps with much more amplifying protocols (PCR based, as used by the Lydall group), one is able to tweak out a difference, but those approaches have their own problems.

We wish to emphasize that both of the points raised by the reviewer do not take away or question the major conclusion that is supported by this figure, namely that rap1-td strains behave much more like wt (or yku70-td) than cdc13-td; thus they experience very limited, if any, degradation into Y'-sequences.

Point 5. For all experiments with td-alleles, Western blots must be shown to ensure that the proteins are indeed degraded.

While we appreciate this suggestion, we would like to refer the reader to data in the published Vodenicharov and Wellinger paper (Mol. Cell 2006) where the requested data on cdc13-td degradation in G1 is shown. In addition, the relevant data on radp1-td is shown in Fig. 5A and also in panels of Figure 6. Furthermore, we would like to point to the fact that we also use phenotypic analyses to show absence of respective proteins, which is much more relevant than just westerns where there is a lower limit of detection. Thus, we feel that the behaviour of these alleles is quite well documented as is.

Minor typos: We thank the reviewer for picking those two typos up and they have been corrected (highlighted in yellow, pages 6 and 7 in the revised manuscript).

