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Pif1- and Exo1-dependent nucleases coordinate checkpoint activation following telomere uncapping

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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 25 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. These referees indeed consider your findings on a Pif1 role in telomere resection potentially interesting and in principle suited for publication in The EMBO Journal. They nevertheless raise a number of specific issues that would need to be adequately addressed prior to eventual publication, to improve the level of mechanistic insight conveyed by the study. As you will see, the points raised by the referees are mostly overlapping and quite specific, and the referees also propose a number of straightforward experimental avenues to address them. I would therefore be happy to give you the opportunity to respond to their concerns in the form of a revised version of the manuscript. Should you be able to adequately clarify the main points through additional experimentation, we should be able to consider such a revised manuscript further for publication. I should however remind you that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

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Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this manuscript, Dewar and Lydall show that the combined inactivation of the Pif1 helicase and Exo1 exonuclease can bypass the essential function of Cdc13 in telomere capping. Furthermore, they show that chromosome end-resection never reaches the subtelomeric Y' element when telomeres are uncapped in exo1 pif1 mutants and that the DNA damage checkpoint is not activated in such mutants, presumably due to a lack of resection. Based on these data, they propose a model in which Pif1 and Exo1 constitute the two major activities involved in resecting telomeres following the inactivation of Cdc13. In the last decade, the Lydall lab has done a great deal of work to identify the nucleolytic activities involved in telomere resection and this manuscript constitutes an interesting addition to their work.

Nevertheless, one must consider that Pif1 is involved in the removal of telomerase from chromosome ends. Therefore, one could imagine that telomerase stays associated with telomeres in the absence of Pif1 to fulfil some of the capping functions that are normally performed by Cdc13. The authors considered this possibility, but disregarded it based on the observation that cdc13-1 tlc1 pif1 mutants grow better at 25 C than cdc13-1 tlc1 mutants (Figure 5A). This reviewer acknowledges this fact and agrees with the authors that Pif1 probably has telomerase-independent activities that inhibit the growth of cdc13-1 mutants. However, the same figure also suggests that an even more important function of Pif1 in inhibiting the growth of cdc13-1 mutants is telomerase-dependent. At 27 C, a temperature at which telomere uncapping becomes more severe in cdc13-1 mutants, it seems that the growth advantage provided by deletion of PIF1 is completely lost in tlc1 mutants. The rest of the analysis performed at higher temperature with the exo1 mutation also supports this notion. Although the model in which Pif1 directly participates in resection of telomeres in the absence of Cdc13 is very attractive, this reviewer is not convinced that the authors can disregard the idea that a telomerase-dependent capping activity contributes to the effect seen in pif1 mutants. Clearly, some additional experiments are required to prove their model. Potentially, conducting an experiment similar to the one presented in figure 5A, but in which the tlc1 mutation would be replaced by a reverse transcriptase-dead allele of EST2 would tell if telomerase activity, or just its presence at telomeres, is responsible for the phenotype observed. Also, looking at Rad53 activation (as presented in figure 3D) or directly measuring resection (as in figure 4B) in pif1 Δ exo1 Δ cdc13-1 tlc1 Δ mutants would allow the authors to draw more definitive conclusions.

In figure 5C, the authors examine the telomeric DNA pattern derived from tlc1Δ cells. They conclude from this figure that tlc1Δ pif1Δ mutants had "undergone a reduction in Y' elements, but had clearly not generated survivor-type telomere structures", and they state in the discussion that these "possibly even constitute a new class of survivor". Considering the difference in loading between lanes 7-8 and 19-20 of figure 5C, it is an overstatement to say that tlc1Δ pif1Δ mutants had undergone a reduction in Y' elements. This could simply be due to the uneven loading. Also, although no Y' amplification can be seen in these mutants (N.B. this, again, might be due to the uneven loading or to the fact that the tlc1Δ pif1Δ mutants are growing poorly and have therefore went through less replication cycles which are likely responsible for the amplification of Y' elements) they fail to mention that the tlc1Δ pif1Δ mutants had clearly generated one telomeric rearrangement typical of type I survivors: all telomeres in these mutants have acquired a terminal Y' element at passage 15 (Supplementary Figure 6: no X-only telomeres are visible when the analysis is done with a TG probe). In my opinion, the tlc1Δ pif1Δ mutants analyzed in figure 5C might represent poorly growing type-I survivors. The authors have to show that these cells are maintained in a Rad51- or Rad52-independent manner if they want to state otherwise.

Finally, it would be interesting to know if some residual activity resects chromosome ends in cdc13-1 pif1Δ exo1Δ at restrictive temperature. To address this, the authors could probe a native slot blot with a telomeric probe or perform in-gel hybridization. This would indicate if the terminal structure

of these mutants is completely normal or slightly altered, like in the ykuΔ mutants.

Additional comments.

- In all the Southern analysis where a Y' probe has been used, X-only telomeres also seem to be visible. Is it due to an incomplete strip of a previous probe or is it that the Y' probe used also picks up some X-sequences?
- Page 15: Fig 5C is referred to as Fig 5D
- Page 16, first paragraph. The authors state that deletion of PIF1 improves the growth of tlc1Δ cells. Isn't it the opposite?
- The authors might want to discuss the possibility that the yku complex is responsible for the recruitment of telomerase in pif1Δ exo1Δ cdc13Δ mutants.

Referee #2 (Remarks to the Author):

Summary:

In the present paper, Dewar and Lydall examine the contribution of Pif1 in the nucleolytic processing of uncapped telomeres. Their results strongly support a role of Pif1 in the processing of uncapped telomeres. Overall, the data are solid and convincing. The finding of Pif1's role in resection of uncapped telomeres is interesting but the paper would greatly benefit from additional mechanistic data.

Major points:

1. Dna2, a 5'-3' DNA helicase and an endonuclease, has been shown to participate in the generation of ssDNA at DSBs. Moreover, DNA2 and genetically interacts with PIF1. Finally, Pif1 and Dna2 collaborate during Okazaki fragment processing. It is essential that the authors test the contribution of DNA2 in the PIF1-dependent processing of uncapped telomeres.

2. The maintenance of viability and telomere repeats in the absence of Cdc13, Pif1 and Exo1 is remarkable but the authors should present a more detailed characterization of this phenotype. For example, it is not clear to this reviewer how telomerase is recruited to telomeres in the absence of Cdc13, is it dependent on Ku?

Minor point:

1. In the abstract and the discussion, the authors stated that "attenuation of DDR ......", a statement that can be misunderstood as providing support for the role of Pif1 at sites other than telomeres. What the authors really mean is attenuation of DDR at uncapped telomeres. They should clarify this point.

Referee #3 (Remarks to the Author):

The goal of this manuscript is to identify nucleases that function at uncapped telomeres following inactivation of Cdc13. Previous work has shown that Exo1 is one of the nucleases that generates ssDNA at telomeres in budding yeast. In this paper, the authors identify Pif1 helicase as a contributor to resection of uncapped telomeres that works in parallel with Exo1 and another nuclease. Importantly, the authors demonstrate that deletion of both PIF1 and EXO1 permits yeast cells to tolerate complete loss of the essential telomere-capping protein Cdc13; that is, their data suggest that the only essential function of Cdc13 is protection of chromosomal ends from Pif1 and Exo1 resection. The authors demonstrate that Pif1 and Exo1-dependent nucleases initiate a potent DNA damage response following Cdc13 inactivation. Pif1 is also determined to play an important role in cells that lack telomerase, as it appears that Pif1 is required for telomerase-independent maintenance of telomeres. Finally, the authors demonstrate that telomerase is crucial for survival of cells lacking Cdc13. Overall, the data presented in the manuscript of Dewar and Lydall represent significant findings that will be of great interest for the readers of EMBO. However, I have a

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number of questions/concerns pertinent to this manuscript. My primary questions are as follows: First of all, the authors propose that telomeres are processed by two (and only two) nucleases including Exo1, and another Pif1-dependent nuclease. I would like to ask whether Sgs1 might also play a role. Bonetti et. al 2009 recently demonstrated that Sgs1 and Sae2 are involved in nucleolytic processing of telomeres, but the results of Dewar and Lydall do not appear to allow for any role of Sgs1 unless it works in the same pathway with Pif1 or Exo1. It is also possible that the end processing taking place in cdc13-1 is somewhat different compared to of the end processing of wild type telomeres. In any case, I am convinced that this issue must be fully explored.

Second, the authors conclude that the effects of pif1Δ are explained by the direct effect of pif1Δ on nucleolytic processing of chromosome ends rather than by stimulating telomerase binding to protect telomeres. This conclusion is made based on the fact that pif1Δ appears to reduce end processing even in tlc1Δ mutants. I believe it is necessary to repeat the same experiment in the est2Δ background, as inactive telomerase may bind the end even in tlc1Δ.

Third, if indeed Pif1 is the helicase that participates in nucleolytic processing of the uncapped telomeres, it is important to determine whether it collaborates with Dna2p (or another protein) during this process. I propose that the authors test the role of Dna2 in this process.

Finally, the authors need to provide a much more detailed discussion of the effect of Pif1 on the formation of Type I and Type II survivors in the tlc1Δ background. Also, the mechanism responsible for formation of the late, unrearranged survivors that appear after passage 15 remains unclear and requires a much more detailed discussion.

1st Revision - authors' response 23 August 2010

Referee #1 (Remarks to the Author):

In this manuscript, Dewar and Lydall show that the combined inactivation of the Pif1 helicase and Exo1 exonuclease can bypass the essential function of Cdc13 in telomere capping. Furthermore, they show that chromosome end-resection never reaches the subtelomeric Y' element when telomeres are uncapped in exo1Δ pif1Δ mutants and that the DNA damage checkpoint is not activated in such mutants, presumably due to a lack of resection. Based on these data, they propose a model in which Pif1 and Exo1 constitute the two major activities involved in resecting telomeres following the inactivation of Cdc13. In the last decade, the Lydall lab has done a great deal of work to identify the nucleolytic activities involved in telomere resection and this manuscript constitutes an interesting addition to their work.

We thank the referee for these positive comments.

Nevertheless, one must consider that Pif1 is involved in the removal of telomerase from chromosome ends. Therefore, one could imagine that telomerase stays associated with telomeres in the absence of Pif1 to fulfil some of the capping functions that are normally performed by Cdc13. The authors considered this possibility, but disregarded it based on the observation that cdc13-1 tlc1 exo1Δ pif1Δ; mutants grow better at 25˚C than cdc13-1 tlc1Δ mutants (Figure 5A). This reviewer acknowledges this fact and agrees with the authors that Pif1 probably has telomerase-independent activities that inhibit the growth of cdc13-1 mutants. However, the same figure also suggests that an even more important function of Pif1 in inhibiting the growth of cdc13-1 mutants is telomerase-dependent. At 27˚C, a temperature at which telomere uncapping becomes more severe in cdc13-1 mutants, it seems that the growth advantage provided by deletion of PIF1 is completely lost in tlc1Δ mutants. The rest of the analysis performed at higher temperature with the exo1Δ mutation also supports this notion. Although the model in which Pif1 directly participates in resection of telomeres in the absence of Cdc13 is very attractive, this reviewer is not convinced that the authors can disregard the idea that a telomerase-dependent capping activity contributes to the effect seen in

pif1Δmutants. Clearly, some additional experiments are required to prove their model. Potentially, conducting an experiment similar to the one presented in figure 5A, but in which the tlc1Δmutation would be replaced by a reverse transcriptase-dead allele of EST2 would tell if telomerase activity, or just its presence at telomeres, is responsible for the phenotype observed. Also, looking at Rad53 activation (as presented in figure 3D) or directly measuring resection (as in figure 4B) in pif1Δexo1Δcdc13-1 tlc1Δ mutants would allow the authors to draw more definitive conclusions.

We thank the referee for these suggestions.

1.1
The idea to perform a similar analysis to that presented in Figure 5A, using a catalytically-dead allele of EST2 is an excellent experimental suggestion. We attempted this experiment but for technical issues were unable to do so within the time frame provided.

We strongly agree with the referee that some of the capping functions that are normally performed by Cdc13 can be performed by telomerase, especially as cdc13 exo1 pif1 mutants depend upon telomerase for viability (Figure 6B). In response to reviews, we repeated the experiments examining the effect of Pif1 and Exo1 on the growth of cdc13-1 tlc1 mutants (Figure 5A) and in parallel performed the same experiment on cdc13-1 est2 mutants (Supplementary Figure 7). Consistent with the refereeís hypothesis, on Page 15 we now note:
"that est2 cdc13-1 and tlc1 cdc13-1 mutants grow worse than cdc13-1 mutants, demonstrating that telomerase contributes to telomere capping following inactivation of Cdc13."

To clarify the data, we now show the growth of cdc13-1 tlc1 mutants at 25∞C as well as 26∞C. This shows that Pif1 and Exo1 both inhibit the growth of cdc13-1 tlc1 and cdc13-1 est2 mutants to a similar extent (26∞C Figure 5A, 25∞C Supplementary Figure 7). This demonstrates that Pif1 and Exo1 have similar effects at uncapped telomeres, even when either component of telomerase is absent. As Exo1 functions as a nuclease at uncapped telomeres, our conclusion that Pif1 also functions as a component of a nuclease activity is robust. This is discussed on Page 15 and we conclude:
"that Pif1 has a telomerase (TLC1, Est2) independent effect at uncapped telomeres."

As requested, we measured Rad53 phosphorylation in cdc13-1 tlc1 mutants. We found that that cdc13-1 tlc1 exo1 pif1 mutants had a phosphorylation defect compared to cdc13-1 tlc1 exo1 mutants (Supplementary Figure 8). On Page 16 we now conclude:
"that Pif1 has a telomerase-independent contribution to checkpoint activation following telomere uncapping."

As discussed further down in 1.4, we have also performed in-gel assays in response to the refereeís comments. In Figure 4F,G we show that the majority of ssDNA generated in the TG repeats at uncapped telomeres is due to Exo1 and that Pif1 has comparably little effect at this locus. This data bolsters our conclusion that Pif1 affects resection of uncapped telomeres independently of telomerase, as the effect of Pif1 is comparably weak in the TG repeats where telomerase should bind. We note this on Page 15 and state:
"It has been suggested that increased levels of telomerase at the telomeres of cdc13-1 pif1 cells shields uncapped telomeres from nuclease activities (Vega et al, 2007). However, this is somewhat inconsistent with our observation that Pif1 has relatively little effect on ssDNA generation in the telomeric TG repeats, where telomerase presumably binds (Figure 4G)."

In figure 5C, the authors examine the telomeric DNA pattern derived from tlc1Δcells. They conclude from this figure that tlc1Δpif1Δmutants had "undergone a reduction in Y' elements, but had clearly not generated survivor-type telomere structures", and they state in the discussion that these "possibly even constitute a new class of survivor". Considering the difference in loading between lanes 7-8 and 19-20 of figure 5C, it is an overstatement to say that tlc1Δpif1Δmutants had undergone a reduction in Y' elements. This could simply be due to the uneven loading.

1.2
We do not consider the loading of lanes 19-20 to be sufficient to account for the loading difference. Lanes 19-20 are indistinguishable in loading from Lane 2, which clearly has much more Yí signal. The Yí signal for Lane 2 is on a par with that of Lanes 7-8, despite Lane 2 being under-loaded

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relative to Lanes 7-8. However, we have better-discussed the telomeres in these cells (as described in point 1.3) and we now refrain from saying that the tlc1 pif mutants "possibly even constitute a new class of survivor". Instead, on Page 18 we conclude:
"that Pif1 is required for the generation of Type I and Type II survivors and that in the absence of Pif1, cells lacking telomerase can improve growth following senescence without adopting typical Type I or Type II survivor structures."

Also, although no Y' amplification can be seen in these mutants (N.B. this, again, might be due to the uneven loading or to the fact that the tlc1Δpif1Δmutants are growing poorly and have therefore went through less replication cycles which are likely responsible for the amplification of Y' elements) they fail to mention that the tlc1Δpif1Δmutants had clearly generated one telomeric rearrangement typical of type I survivors: all telomeres in these mutants have acquired a terminal Y' element at passage 15 (Supplementary Figure 6: no X-only telomeres are visible when the analysis is done with a TG probe). In my opinion, the tlc1Δpif1Δmutants analyzed in figure 5C might represent poorly growing type-I survivors. The authors have to show that these cells are maintained in a Rad51- or Rad52-independent manner if they want to state otherwise.

1.3
We thank the referee for this insightful comment. The acquisition of terminal Yí elements has now been noted both in our results and our discussion. On page 18 we say:
"that tlc1 pif1 and tlc1 pif1 exo1 mutants resembled Type I survivors in that our TG probe did not detect any individual telomeres further up the gel (marked by arrows, compare lanes 7-8 to 19-20 and lanes 11-12 to 23-24, Supplementary Figure 10), indicating that all telomeres in these strains had acquired a terminal Yí fragment."

Finally, it would be interesting to know if some residual activity resects chromosome ends in cdc13-1 pif1Δexo1Δat restrictive temperature. To address this, the authors could probe a native slot blot with a telomeric probe or perform in-gel hybridization. This would indicate if the terminal structure of these mutants is completely normal or slightly altered, like in the ykuΔmutants.

1.4
We thank the referee for suggesting this experiment. We have performed in-gel assays to address the refereeís concerns, using the increased ssDNA seen in the TG repeats of yku70 mutants as a positive control for the assay.

In Figure 4F-G we demonstrate that cdc13-1 pif1 exo1 mutants at restrictive temperature do generate ssDNA 2 hours after telomere uncapping, as the referee suggests but that this is no longer detectable by 4 hours. This is discussed on Page 14 and we conclude:
"cdc13-1 exo1 pif1 mutants generate limited, transient ssDNA that is insufficient to stimulate checkpoint activation"

In Figure 7C-D we demonstrate, as the referee suspected, that exponentially-dividing cdc13-1 exo1 pif1 mutants at the restrictive temperature and cdc13 exo1 pif1 mutants generate similar levels of ssDNA to yku70 mutant. As cdc13 exo1 pif1 mutants have gone through many cell divisions with uncapped telomeres, this argues that they do not accumulate ssDNA over time, as would be expected if some residual activity resects chromosome ends. This is discussed on pages 22-23 and we conclude:
"that continued growth following telomere uncapping in exo1 pif1 mutants does not lead to ssDNA accumulation. This suggests that no residual nuclease activities continue to resect uncapped telomeres in the absence of Pif1 and Exo1."

Additional comments.

- In all the Southern analysis where a Y' probe has been used, X-only telomeres also seem to be visible. Is it due to an incomplete strip of a previous probe or is it that the Y' probe used also picks up some X-sequences?

1.5
The Yí probe does appear to cross-hybridize to X-only sequences. We have clarified this in the text and on Page 9 state:

"The Yí probe contained G-rich sequences and weakly cross-hybridized to telomeres which did not contain Yí sequences, so we also probed for TG repeat sequences to robustly detect telomeres that lacked Yí elements"

- Page 15: Fig 5C is referred to as Fig 5D

1.6
We thank the reviewer and have amended accordingly.

- Page 16, first paragraph. The authors state that deletion of PIF1 improves the growth of tlc1Δcells. Isn't it the opposite?

1.7
We originally stated "the absence of Pif1 permits tlc1 cells to maintain relatively normal telomere structure and improve their growth without gross alterations in telomere structure." The improvement in growth we referred to is that which occurs following senescence. We did not want to use the word ërecoverí because pif1 tlc1 mutants still show a very clear and severe growth defect. This section has been altered in response to point 1.2 and we have further added the words ëfollowing senescenceí to clarify. On Page 18 we now say:
"Pif1 is required for the generation of Type I and Type II survivors and that in the absence of Pif1, cells lacking telomerase can improve growth following senescence without adopting typical Type I or Type II survivor structures."

- The authors might want to discuss the possibility that the yku complex is responsible for the recruitment of telomerase in pif1Δexo1Δcdc13Δmutants.

1.8
We thank the referee for this suggestion. We have now not only discussed this but also tested it and demonstrated that Yku70 is required for the viability of pif1 exo1 cdc13 mutants (Figure 6C, Supplementary Figure 11). On page 26, in the discussion, we now say:
"We have demonstrated that attenuation of the DDR, by elimination of Pif1 and Exo1 permits telomere maintenance in a Cdc13-indpendent but telomerase and Ku-dependent manner. This is surprising because Cdc13 is considered crucial for efficient recruitment of telomerase and thus to prevent senescence (Nugent et al, 1996). We propose that in the absence of Cdc13, Yku80 binds TLC1, the telomerase RNA, to help recruit telomerase to the telomere (Peterson et al, 2001)."

Referee #2 (Remarks to the Author):

Summary:

In the present paper, Dewar and Lydall examine the contribution of Pif1 in the nucleolytic processing of uncapped telomeres. Their results strongly support a role of Pif1 in the processing of uncapped telomeres. Overall, the data are solid and convincing. The finding of Pif1's role in resection of uncapped telomeres is interesting but the paper would greatly benefit from additional mechanistic data.

Major points:

1. Dna2, a 5'-3' DNA helicase and an endonuclease, has been shown to participate in the generation of ssDNA at DSBs. Moreover, DNA2 and genetically interacts with PIF1. Finally, Pif1 and Dna2 collaborate during Okazaki fragment processing. It is essential that the authors test the contribution of DNA2 in the PIF1-dependent processing of uncapped telomeres.

2.1
We have examined the effect of dna2 in a cdc13-1 pif1 background to test the effect of Dna2 on Pif1-independent processing of uncapped telomeres (Suppelementary Figure 2). We show that at 28.5∞C, cdc13-1 pif1 mutants can grow, while cdc13-1 pif1 dna2 mutants cannot. Thus, Dna2 plays a protective role, independent of Pif1, during the resection of uncapped telomeres. This is

discussed on Page 8 and we conclude:
"that Exo1 inhibits the growth of cdc13-1 mutants with uncapped telomeres while Sgs1 and Dna2 contribute to the vitality of such cells. Therefore we chose to focus on the roles of Pif1 and Exo1 at uncapped telomeres."

We considered that since Dna2 is both a helicase and a nuclease and that the helicase role of Dna2 might be protective to telomeres while the nuclease might be detrimental. However, we found that overexpressing a helicase-dead or wild type copy of DNA2 was able to complement the loss of viability caused by deleting DNA2 in cdc13-1 pif1 mutants. The same complementation was not observed when over-expressing a nuclease-dead allele of DNA2. This data has been left out of the manuscript, as we did not feel it contributed to a cohesive story, but we can include the data if the referees feel it necessary.

Unfortunately, we are unable to test the contribution of Dna2 to Pif1-dependent processing of uncapped telomeres. DNA2 is an essential gene and so null mutations cannot be examined without deleting Pif1, which eliminates Pif1-dependent processing of uncapped telomeres. A temperature-sensitive allele of DNA2 (dna2-1) exists but this leads to replication defects, causing arrest at S phase, while uncapped telomeres undergo resection primarily at metaphase.

2. The maintenance of viability and telomere repeats in the absence of Cdc13, Pif1 and Exo1 is remarkable but the authors should present a more detailed characterization of this phenotype. For example, it is not clear to this reviewer how telomerase is recruited to telomeres in the absence of Cdc13, is it dependent on Ku?

2.2
We have now demonstrated that Yku70, Rad52 and telomerase are required for the viability of pif1 exo1 cdc13 mutants but Pol32 is not (Figure 6C, Supplementary Figure 11, Supplementary Figure 12).

On page 21, in the results we now say:
"We also found that Yku70 (a component of the Ku complex, which binds TLC1 to aid in recruitment of telomerase to the telomere) and Rad52 (required for homologous recombination and the generation of Type I and Type II survivor telomere structures) were required for the viability of cdc13 pif1 exo1 mutants (Figure 6C). However, we found that Pol32 (subunit of Polymerase , required for the generation of Type I and Type II survivor telomere structures) was dispensable for the viability of cdc13 pif1 exo1 mutants (Figure 6C), although elimination of Pol32 did reduce the frequency at which cdc13 pif1 exo1 mutants were able to lose the pURA3[CDC13] (Supplementary Figure 12). We conclude that cdc13 pif1 exo1 mutants are distinct from Type I and Type II survivors, as they do not require Pol32, and their telomeres are maintained through a combination of homologous recombination, Ku and telomerase activity."

On page 26-27 in the discussion we now say:
"elimination of Pif1 and Exo1 permits telomere maintenance in a Cdc13-indpendent but telomerase and Ku-dependent manner. This is surprising because Cdc13 is considered crucial for efficient recruitment of telomerase and thus to prevent senescence (Nugent et al, 1996). We propose that in the absence of Cdc13, Yku80 binds TLC1, the telomerase RNA, to help recruit telomerase to the telomere (Peterson et al, 2001). The requirement for Rad52 for the survival of cdc13 exo1 pif1 mutants is surprising. It will be interesting to investigate whether telomeric repeats from extremely long telomeres in cdc13 exo1 pif1 mutants (Figure 7B) can be distributed to shorter telomeres by homologous recombination, thus preventing short telomeres from becoming critically short."

We have also characterized the pif1 exo1 cdc13 mutants further by performing an in-gel assay in Figure 7C-D to demonstrate that pif1 exo1 cdc13 mutants do have some detectable telomeric ssDNA, but at a level comparable to yku70 mutants at low temperature, which do not undergo extensive resection or cell cycle arrest.

On page 23 of the results we note:
"cdc13 exo1 pif1 mutants from Passage 1 (approximately 50 population doublings with uncapped telomeres) generated comparable levels of ssDNA in the TG repeats to a yku70 mutant (Figure 7C,D)."

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Minor point:

1. In the abstract and the discussion, the authors stated that "attenuation of DDR ......", a statement that can be misunderstood as providing support for the role of Pif1 at sites other than telomeres. What the authors really mean is attenuation of DDR at uncapped telomeres. They should clarify this point.

2.3
We have added the words ëat uncapped telomeresí to clarify and now conclude the abstract with:
"Thus, attenuation of the DDR at uncapped telomeres can circumvent the need for otherwise-essential telomere capping proteins."
On Page 26, as part of the discussion, we say:
"We have demonstrated that attenuation of the DDR at uncapped telomeres, by elimination of Pif1 and Exo1 permits telomere maintenance in a Cdc13-indpendent but telomerase and Ku-dependent manner."

Referee #3 (Remarks to the Author):

The goal of this manuscript is to identify nucleases that function at uncapped telomeres following inactivation of Cdc13. Previous work has shown that Exo1 is one of the nucleases that generates ssDNA at telomeres in budding yeast. In this paper, the authors identify Pif1 helicase as a contributor to resection of uncapped telomeres that works in parallel with Exo1 and another nuclease. Importantly, the authors demonstrate that deletion of both PIF1 and EXO1 permits yeast cells to tolerate complete loss of the essential telomere-capping protein Cdc13; that is, their data suggest that the only essential function of Cdc13 is protection of chromosomal ends from Pif1 and Exo1 resection. The authors demonstrate that Pif1 and Exo1-dependent nucleases initiate a potent DNA damage response following Cdc13 inactivation. Pif1 is also determined to play an important role in cells that lack telomerase, as it appears that Pif1 is required for telomerase-independent maintenance of telomeres.
Finally, the authors demonstrate that telomerase is crucial for survival of cells lacking Cdc13. Overall, the data presented in the manuscript of Dewar and Lydall represent significant findings that will be of great interest for the readers of EMBO.

We are pleased that this referee found our paper so interesting.

However, I have a number of questions/concerns pertinent to this manuscript. My primary questions are as follows:
First of all, the authors propose that telomeres are processed by two (and only two) nucleases including Exo1, and another Pif1-dependent nuclease. I would like to ask whether Sgs1 might also play a role. Bonetti et. al 2009 recently demonstrated that Sgs1 and Sae2 are involved in nucleolytic processing of telomeres, but the results of Dewar and Lydall do not appear to allow for any role of Sgs1 unless it works in the same pathway with Pif1 or Exo1. It is also possible that the end processing taking place in cdc13-1 is somewhat different compared to of the end processing of wild type telomeres. In any case, I am convinced that this issue must be fully explored.

3.1

We did not intend to propose that telomeres in general are processed by only two nucleases. We agree with the referee that the processing of uncapped telomeres is very different to that of wild type telomeres. This is evidenced by the importance of Pif1 in the processing of uncapped telomeres that we have demonstrated here compared to the dispensability of Pif1 for the processing of shortened telomeres as seen in Bonetti et al. (2009).

Since the submission and review of this manuscript, another manuscript from our lab dealing with the role of Sgs1 at uncapped telomeres has been published (Ngo and Lydall (2010) PLoS Genet). This is now referenced in our discussion, where we highlight the complexity of nuclease regulation at DNA Double Strand Breaks and uncapped telomeres. On page 25 of the discussion, we now say:
"DSBs that can be repaired by homologous recombination and DSB-induced shortened telomeres

are processed by nucleases dependent upon Sgs1/Dna2, Exo1 and MRX/Sae2 (Gravel et al, 2008; Mimitou & Symington, 2009; Zhu et al, 2008). Other work recently published from our lab demonstrates that Sgs1 also contributes to resection of uncapped telomeres, but elimination of Sgs1 and Exo1 is insufficient to prevent the resection of uncapped telomeres in cdc13-1 mutants (Ngo & Lydall, 2010). The work presented here demonstrates that elimination of Pif1 and Exo1 prevents resection of uncapped telomeres in cdc13-1 mutants. However, at DSBs that can be repaired by homologous recombination or at DSB-induced shortened telomeres, Pif1 has little effect on resection (Bonetti et al, 2009; Zhu et al, 2008). Interestingly, Pif1 has been shown to play a critical role repair of DSBs where Break-Induced Replication (BIR) is the main repair pathway (Chung et al, 2010). A major challenge will be to determine which substrates are exposed at DSBs, shortened telomeres and uncapped telomeres and how nuclease activities are coordinated to process them."

Thus, concordant with the refereeís suggestion, Sgs1 does play a role in the processing of uncapped telomeres (Ngo and Lydall (2010) PLoS Genet). In Supplementary Figure 2 we show that the sgs1 mutation inhibits growth of both cdc13-1 and cdc13-1 pif1 mutants, indicating that Sgs1 acts in a different pathway to Pif1. We have, of coursed, considered examining the role of Sgs1 as part of this study. However, given that Exo1 and Sgs1 have very similar roles at DSBs but very different roles at uncapped telomeres and given that Pif1 has a completely novel role in resection at uncapped telomeres, which it does not have at DSBs we feel this would be far too complex to deal with in a single manuscript. To make this clear, on Page 8 we now say:
"We conclude that Exo1 inhibits the growth of cdc13-1 mutants with uncapped telomeres while Sgs1 and Dna2 contribute to the vitality of such cells. Therefore we chose to focus on the roles of Pif1 and Exo1 at uncapped telomeres."

Second, the authors conclude that the effects of pif1Δ are explained by the direct effect of pif1Δ on nucleolytic processing of chromosome ends rather than by stimulating telomerase binding to protect telomeres. This conclusion is made based on the fact that pif1Δ appears to reduce end processing even in tlc1Δ mutants. I believe it is necessary to repeat the same experiment in the est2Δ background, as inactive telomerase may bind the end even in tlc1Δ.

3.2
As suggested, we have repeated this experiment in an est2 background. Supplementary Figure 7 shows that est2 cdc13-1 pif1 and est2 cdc13-1 exo1 mutants show improved growth compared to est2 cdc13-1 mutants and that the double est2 cdc13-1 pif1 exo1 mutant grows better than either of the single mutants, demonstrating that Pif1 can function at uncapped telomeres even in the absence of telomerase (Est2). On Page 15 we now conclude:
"that Pif1 has a telomerase (TLC1, Est2) independent effect at uncapped telomeres."

Additionally, as discussed in 1.1, we have also demonstrated that Pif1 contributes to the Rad53 phosphorylation seen following telomere uncapping in tlc1 cdc13-1 exo1 mutants (Supplementary Figure 8).

Third, if indeed Pif1 is the helicase that participates in nucleolytic processing of the uncapped telomeres, it is important to determine whether it collaborates with Dna2p (or another protein) during this process. I propose that the authors test the role of Dna2 in this process.

3.3
We agree that the role of Dna2 at uncapped telomeres is extremely important to elucidate. However, as discussed in 2.1 we do not feel it is not feasible to assess the contribution of Dna2 to Pif1-dependent nucleolytic processing. We have, however assessed the Pif1-independent roles of Dna2 at uncapped telomeres and found Dna2 to have a protective role (Supplementary Figure 2). As mentioned in 3.1 we state our logic for not taking our investigation of Dna2 any further, saying on Page 8:
"Exo1 inhibits the growth of cdc13-1 mutants with uncapped telomeres while Sgs1 and Dna2 contribute to the vitality of such cells. Therefore we chose to focus on the roles of Pif1 and Exo1 at uncapped telomeres."

Finally, the authors need to provide a much more detailed discussion of the effect of Pif1 on the formation of Type I and Type II survivors in the tlc1Δ background. Also, the mechanism responsible for formation of the late, unrearranged survivors that appear after passage 15 remains unclear and

requires a much more detailed discussion.

3.4
The telomeric structures are now described in more detail in the results. On Page 18 we now say: "We noted that tlc1 pif1 and tlc1 pif1 exo1 mutants did resemble Type I survivors in that our TG probe did not detect any individual telomeres further up the gel (marked by arrows, compare lanes 7-8 to 19-20 and lanes 11-12 to 23-24, Supplementary Figure 10), indicating that all telomeres in these strains had acquired a terminal Yí fragment. However, the terminal fragments of tlc1 pif1 and tlc1 pif1 exo1 were even shorter than those of Type I survivors and they had undergone a reduction, not an amplification in Yí elements, clearly distinguishing them from typical Type I survivors (compares lanes 19-20, 23-24 to lanes 21-22, Figure 5D). We conclude that Pif1 is required for the generation of Type I and Type II survivors and that in the absence of Pif1, cells lacking telomerase can improve growth following senescence without adopting typical Type I or Type II survivor structures."

We also discuss the possible mechanism on Page 24 and say:
"Pif1 contributes to the vitality of cells lacking telomerase, both before and after recovery from senescence (Figure 5B). Interestingly pif1 cells improve their growth following senescence without adopting typical survivor-like telomeric DNA structures (Figure 5C). Usually following senescence, survivors are generated by homologous-recombination- and BIR-dependent alterations in telomere structure (Lydeard et al, 2007; Teng & Zakian, 1999). If BIR is eliminated, cells lacking telomerase senesce and undergo a complete loss in viability (Lydeard et al, 2007). The relatively unaltered telomere structure and poor growth following senescence in cells lacking Pif1 and telomerase is consistent with the impaired BIR seen in cells lacking Pif1 (Chung et al, 2010). Therefore, reduced BIR in pif1 cells may be sufficient to maintain comparatively normal telomere structure in telomerase-deficient cells but insufficient to permit the typical amplification of Yí elements or terminal TG repeats seen in survivors. The absence of telomeric repeat amplification could prevent these cells from achieving the high levels of post-senescence growth seen in other telomerase-deficient mutants."

2nd Editorial Decision 06 September 2010

Thank you for submitting your revised manuscript for our consideration. Referees 1 and 3 have now evaluated it once more, and I am pleased to inform you that both of them consider the manuscript significantly improved and the majority of issues adequately addressed. As you will see, there is nevertheless one of the original concerns remaining to be more satisfactorily addressed, regarding the possibility of Pif1 deletion acting via telomerase rather than directly on resection. As an alternative to the technically challenging experiments originally requested to address this, referee 1 is now suggesting a more straightforward genetic strategy, and if at all feasible, I would like to strongly encourage you to conduct this last experiment, and to get a re-revised manuscript including these data and a brief response back to us as soon as possible. Should you have any further questions in this regard, please of course do not hesitate to contact me for further discussions.

Thank you and I am looking forward to receiving your final version.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Comments on the revised version of "Pif1- and Exo1-dependent nucleases coordinate checkpoint activavtion following telomere uncapping.

In the revised version of their manuscript Dewar and Lydall added several experiments to address most of the concerns this reviewer had. However, an important issue raised by reviewer #3 and myself is left unresolved. Because Pif1 is involved in the removal of telomerase from telomeres, it is conceivable that the effect of PIF1 deletion on the growth of cdc13 mutants is due to its role on telomerase rather than because it has a direct effect on chromosome end resection. Unfortunately, the experiment this reviewer suggested could not be performed due to technical difficulties and the additional experiment performed to address the same concern raised by reviewer #3 is, in my opinion, not adequate. What is needed is a direct measurement of DNA resection in cdc13-1 tlc1 pif1 exo1 mutants. If, as depicted in figure 8, the major role of Pif1 is at the level of DNA processing, one would expect chromosome end degradation in cdc13-1 TLC1 pif1 exo1 to be more or less equal to the one observed in cdc13-1 tlc1 pif1 exo1 mutants. Since these strains are already available to the authors (Fig. 5), and because that have robust assays to detect chromosome end resection they should be able to perform this experiment.

Referee #3 (Remarks to the Author):

The goal of this manuscript is to identify nucleases that function at uncapped telomeres following inactivation of Cdc13. Previous work has shown that Exo1 is one of the nucleases that generates ssDNA at telomeres in budding yeast. In this paper, the authors identify Pif1 helicase as a contributor to resection of uncapped telomeres that works in parallel with Exo1 and another nuclease. Importantly, the authors demonstrate that deletion of both PIF1 and EXO1 permits yeast cells to tolerate complete loss of the essential telomere-capping protein Cdc13; that is, their data suggest that the only essential function of Cdc13 is protection of chromosomal ends from Pif1 and Exo1 resection. The authors demonstrate that Pif1 and Exo1-dependent nucleases initiate a potent DNA damage response following Cdc13 inactivation. Pif1 is also determined to play an important role in cells that lack telomerase, as it appears that Pif1 is required for telomerase-independent maintenance of telomeres. Finally, the authors demonstrate that telomerase is crucial for survival of cells lacking Cdc13. Overall, the data presented in the manuscript of Dewar and Lydall represent significant findings that will be of great interest for the readers of EMBO.
Importantly, the authors went a long way to address the reviewer's comments and in my opinion did it successfully.
Therefore, I wholeheartedly support publication of the manuscript "Pif1- and Exo1-dependent nucleases coordinate checkpoint activation following telomere uncapping" by Dewar and Lydall in EMBO journal.

2nd Revision - authors' response 27 September 2010

Thank you for your letter of 6th September and for the further consideration of our manuscript. We were pleased to find that Referee #3 now fully endorses our manuscript and that Referee #1 felt most of their concerns were addressed.
As you requested, we have now performed the additional experiment requested by Referee #1. In Supplementary Figure 8B-C we now show that resection of cdc13-1 tlc1 exo1 pif1 mutants is reduced compared to cdc13-1 tlc1 exo1 mutants and is similar to cdc13-1 TLC1+ exo1 pif1 mutants. We also show that tlc1 cdc13-1 pif1 mutants generate less ssDNA following telomere uncapping than tlc1 cdc13-1 mutants. Both of these experiments demonstrate a clear role for Pif1 in the resection of uncapped telomeres in cells lacking telomerase.
The experiment requested by Referee #1 has caveats. Our primary concern is that resection of uncapped telomeres is a cell cycle regulated process and occurs primarily at metaphase.

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In Supplementary Figure 3D we show that 30-40% of cells in tlc1 cdc13-1, tlc1 cdc13-1 pif1 and tlc1 cdc13-1 exo1 cells at 23∞C are at metaphase. Thus, we cannot rule out the possibility that the differences in ssDNA we have measured are due to altered kinetics of accumulation at metaphase following telomere uncapping. This has been noted in the manuscript.
Following the first round of review, Referee #1 suggested that 'looking at Rad53 activation (as presented in figure 3D) or directly measuring resection (as in figure 4B) in pif1 exo1 cdc13-1 tlc1 mutants would allow the authors to draw more definitive conclusions.' In our previous submission we included measurements of Rad53 activation in telomerase-deficient cdc13-1 mutants (now given in Figure 8A). We somewhat are concerned that Referee #1 then required us to measure Rad53 activation and resection. However, despite this concern we have now done all that was asked of us to completely address the concerns of Referee #1.

Acceptance letter 29 September 2010

Thank you for submitting your re-revised manuscript including the remaining requested experiment, whose results I have now reviewed in light of the previous reviewer comments. I am happy to inform you that there are no further objections against acceptance, and that we should now be able to swiftly proceed with its production and publication.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

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