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Mammalian DNA2 helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity

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Editor: Hartmut Vodermaier

1st Editorial Decision 28 November 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the comments of three expert referees in the field, which you will find copied below. As you will see, the reviewers appreciate that your findings on mammalian DNA2 telomeric and G4 DNA-related roles are of interest and potentially important. However, they also raise a number of substantive concerns that currently preclude publication, as they have to potential of confounding some of the main conclusions. Should you be able to decisively address these concerns through additional experimental work, we would be willing to consider a revised version of the manuscript further for publication. In this respect, it will however be absolutely essential to improve the technical quality (in particular of those experiments criticized by referees 1 and 2 such as ChIP data, IF data, controls...) to these referees' satisfaction. Furthermore, I agree with referee 3's criticism regarding the writing, presentation, and referencing of the manuscript, which will necessitate extensive re-writing especially of introduction (to clearly state the current knowledge of the field, based on citation of the relevant primary literature already at this point) and discussion (to better place your current findings and their potential significance in the context of the current knowledge about the roles and resolution of telomeric G4 DNA). On the other hand, I feel that some of the further-reaching experimental proposals of referee 3 (such as points 4, 6, 9) may be beyond the scope of the current study and would not need to be included in the course of this revision. Regarding referee 3's points 8 and 11, relating to the potential of genome-wide rather than telomere-specific problems, additional data may be required, unless already existing evidence should allow you to rule out these possibilities as unlikely scenarios.

When revising the manuscript, please keep in mind that it is our policy to allow a single round of major revision only, and that it will therefore be essential to diligently and thoroughly answer to all the points raised by the reviewers at this stage (including those mentioned above where we consider further experiments beyond the scope of the revision). Please also bear in mind that your letter of response to the referees' comments will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Thank you again for the opportunity to consider this work for publication, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

REFEREE COMMENTS

Referee #1

In this manuscript Lin et al explore the role of DNA2 in telomere replication. The authors suggest that a portion of DNA2 colocalizes with telomeres and that DNA2 coprecipitates with TRF1 and TRF2. The authors then generate a deletion of DNA2 in the mouse and find that DNA2 heterozygotes suffer from fragile telomeres at leading and lagging telomeres, TSCE, sister telomere associations and are sensitive to aphidicolin.
Then the authors conduct a thorough in vitro analysis of the G4 unwinding capabilities of DNA 2 and find that the DNA2 nuclease activity efficiently acts on a number of G4 substrates. Accordingly, G4 stabilizers strengthen the phenotype in cells.
Then the authors move back to mouse cells and find an elevated level of TIF as well as anaphase bridges and tetraploid/aneuploid chromosome sets.
Analysis of the deletion-animals revealed a high incidence of aneuploidy and associated cancers, and that cells and cancer cells in the animals had shorter telomeres.
In general this is a very interesting manuscript with a high potential. The generation of the DNA2 knockout mouse and the resulting telomere phenotypes is interesting, as well as the synergy with G4 stabilizers. Many of the experiments are convincing and very well executed.
The one real problem lies in Figure 1, as outlined below. It almost seems as if these experiments were done independently of the rest, since they are of such lower quality and lack controls. Once this problem has been addressed, the findings will be interesting to a wide audience.

Figure 1:
The ChIP is not convincing and needs repetition, quantification and error bars. Similarly The IF is of very poor quality and colicalization could be a result of random overlap, and need quantification.
Along the same lines, the IPs need further standard controls, such as input, a control with another flag tagged protein that does not interact with DNA2, antibody only, beads only...
I indicated this manuscript as of low technical quality for Figure 1 only, the other figures are much better.

Figures 2, 3, 4:
The mouse deletion and telomere analysis here is far more convincing and very well executed. Similarly, the in vitro experiments are beautiful.

Figure 5:

The data presented here are convincing due to the quantification. The images are still poor quality though. Also, this figure could be combined with Fig 1.

Figures 6, 7:
These approaches are again highly convincing and well executed.

Referee #2

DNA2 is a nuclease/helicase that has been demonstrated to play several roles in promoting efficient DNA replication and repair. This manuscript describes an additional role for this protein, in promoting DNA replication at telomeres. In vitro experiments convincingly demonstrate that the nuclease activity of DNA2 is able to cleave within and beside G-quadruplex structures formed in telomeric substrates resembling replication forks and flaps. Mice heterozygous for DNA2 demonstrate multiple telomere abnormalities, providing in vivo evidence for a function of this protein in promoting replication through telomeric DNA.

These data provide an important advance in understanding the biological roles of this multifunctional protein. They also provide additional evidence for the emerging view that telomeric DNA, due to its G-rich nature, presents special difficulties for the DNA replication machinery, and that multiple proteins are involved in resolving these difficulties. The paper is clearly and concisely written, and the in vitro experiments in particular are robust and convincing.

My major concern is with the technical quality of many of the cell staining experiments, casting some doubt on the conclusions drawn from those experiments. Specifically, my concerns are as follows:
1) The colocalizations in Figure 1B are not at all convincing, though a higher resolution version of the figure might show some improvement. The image is highly pixelated, to the extent that most of the foci appear square. In addition, all the colocalizing foci only partially overlap. The DNA2 signal also appears oversaturated. Finally, colocalization is always more convincing if multiple Z-stacks are imaged, to rule out coincidental overlapping signals in different planes of the cell.
2) A siRNA control was provided to verify western blot bands using the DNA2 antibody (Figure S4); similar control experiments should be provided to verify that the antibody gives specific signals in ChIP and IF experiments.
3) Commercial antibodies to TRF1 are notoriously unreliable, so similar siRNA controls should be performed to verify IF signals from the TRF1 and TRF2 antibodies. In human cells in particular (Figure S1), the staining from DNA2, TRF1 and TRF2 antibodies seems to be largely cytoplasmic or perinuclear.
4) Staining for telomeric DNA in figure 5A is also poor quality, with a lot of diffuse background.
5) In Figure 3A, how are sister telomere associations distinguished from chromosomes which have lost the telomeric DNA from a single chromatid?
6) The lower right panel of Figure 3D demonstrates that a high proportion of chromosome ends have undergone T-SCE events, but I don't understand why a similarly high proportion is not observed in the panel above, which uses the same technique.
7) In Figure 7C, how are the boundaries of individual nuclei identified?

A more minor comment involves the synthetic G-quadruplex substrates: in Materials and Methods, it is stated that native gel electrophoresis was performed to verify formation of the folded structure. These gels should be shown, possibly as a supplementary figure.

Referee #3

Mammalian DNA2 cleaves telomeric G4 to alleviate replication stress and maintain telomere integrity

The authors examined the function of Dna2 at mammalian telomeres. In their study they report a novel function of Dna2 for telomeric replication by cleaving G4 DNA. They showed that Dna2 localizes to telomeres by immunostaining and co-IP of two prominent members of the shelterin complex (TRF1 and TRF2). In further studies they evaluated the replication changes and DNA damage signal at telomeres in the presence and absence of Dna2. These studies were accompanied by in vitro analysis showing the Dna2 can cleave intramoleculare G4. Consistently Dna2-deficient MEFs showed fragile telomeres after treatment with TMPyP4, a G4 stabilization agent. They suggest a model in which Dna2 nuclease function is essential for promoting telomeric replication by cleaving G4 structures. Consequently Dna2 removes replication stress at telomeres and by this preserved genome stability.
The question about telomere replication and the consequences for genome integrity is a very important research topic for many telomere and cancer scientists. The presented data are very promising but lacks crucial controls and also direct support for their model. G4 DNA is a very controversially discussed secondary DNA structure and it is not clear weather G4 forms in vivo and what its function is. It seems that their experiments take it as a given fact that G4 structure forms at telomeres, but they do not say what, in their opinion, the function of G4s at telomeres is (which is to date not clear). Their data on Dna2 localization are clear and promising, but their conclusion and connection to G4 structures are not well documented by their results. Their interpretation of Dna2 function during telomere replication by cleaving G4 is not convincingly supported by their data. Many questions are still unresolved and confusing for the reader, e.g.: Is the defect they observed really mainly due to telomere replication or an indirect effect of the multifunctional nature of Dna2 (mitochondria, Okazaki fragment maturation); also how does their data fit into G4 function and telomerase activity? What specific function could G4 have for telomere maintenance? Questions on telomerase function are especially interesting, because there are reports showing that telomerase activity is inhibited by G4 (Zahler 1991) and on the other hand that intramolecular G4 can influence telomerase activity in a positive way (Oganesian 2006, 2007). In addition an alternative model apart from G4 needs to be discussed, because there is no clear evidence that G4 form at telomeres in vivo. In summary a more critical view on their data, controls, and interpretation would be beneficial for the paper.

Issues of concern:

1. The introduction is very well written, but lacks an introduction on G4 structures and the introduction to Dna2 switches between results from yeast and humans.
2. Furthermore, the connection of telomere maintenance and G4 formation is completely missing and what is written is confusing and lacks current and past important in vivo and in vitro data, e.g. Zaugh 2005, Paeschke et al 2005, Bonetti 2009, Smith 2011, and others.
3. Many helicases, which contribute to genome stability at telomeres, can unwind G4 structures. In the manuscript they mention that WRN and BLM helicase unwind G4 structures in a different manner than Dna2. This is correct but also not surprising, because Dna2 cleaves G4 and WRN and BLM unwinds the structure. In addition WRN and BLM are not the only helicases that unwind G4; Pif1, FANCJ, RTEL, and other helicases have a similar unwinding potential. The current research suggests that different helicases might act at different G4s. A detailed discussion would be essential to thoroughly evaluate their Dna2 data.
4. The interesting data about Rqh1 sumoylation and its connection to telomere replication is missing from their discussion. It would also be interesting to see, if there is a connection between Dna2 and Rqh1 during telomere replication. Sumoylation of Rqh1 is suggested to affect telomere binding and influence telomere replication allowing unwinding of G4s.
5. The current manuscript completely lacks a discussion about the difference between inter and intramolecular G4. From past research it is known that intermolecular G4 and intramolecular G4 are regulated differently and are proposed to have different function. Can Dna2 cleave both structures? What is their specific binding constant (Kd). Detailed in vitro analyses of Dna2 would be useful to evaluate their data. Also control substrates would be useful.
6. They show convincingly that Dna2 interacts with TRF1 and TRF2, but their story stops short after this very interesting finding. How is the interaction facilitated and why? Is there a consequence for telomere replication? This could be easily addressed by different in vitro assays or pull downs.
7. There are other telomere-binding proteins known that affect G4 structure like Pot1, Mre11, Cdc13, WRN and BLM, and others. How do their function fit into the current model?
8. Dna2 has many different functions. The best-studied role is during Okazaki fragment maturation. Could it be that the observed genome stability defects are a consequence of mis-regulated Okazaki

fragment maturation genome-wide. Experiments addressing this concern are important to support their hypothesis.
9. Dna2 is also essential in the mitochondria. The major protein population of Dna2 is in the mitochondria. It is well known that mitochondria defects contribute greatly to the overall phenotype of the cell. To address this question it would be good to analyze the mitochondrial defect in detail in order to compare it to the nuclear function.
10. G-quadruplexes are a very important and currently highly discussed topic. Many scientists are trying to address the question if G-quadruplex motifs can form G-quadruplex structures in vivo and if so, what their function might be. It would be beneficial for the reader to know what the function of G4 structures are at telomeres and if the authors think that G4 form always at telomeres.
11. TMPyP4 is not an ideal G4 stabilization ligand. TMPyP4 is not specific for telomeres, it is also used in general to stabilize G4 structure. Furthermore recent publications show that it can also bind and stabilize duplex DNA. The observed effect could also be that many G4 structures (in human over 300 000) are stabilized and effect replication events or that the stabilized duplex DNA acts differently during DNA replication. G4 ligands are a nice addition for some analyses, but alone not proof of G4 formation. The ligand can even induce the formation of such structures. A more critical evaluation here is essential.

Minor comment

Introduction:
Several Refs. are cited wrong or are missing: e.g. in the introduction a REF (no review) showing G4 dependent block of telomere replication would be useful. The later cited articles Sfeir 2009 do not show this, they only argue in one sentence that G4 structure might help to understand their data, Bochman et al. is a review article and Vannier et al. is also not showing direct evidence that G4 block DNA replication at telomeres.

One of the last sentences on Page 4 has grammar issues: " ....how do nucleases/helicases resolve telomeric G4, is G4 unwindig is the only way....." is not correct.

G4 is used before the G-quadruplex (G4) is introduced.

Results:
qPCR analysis of their ChIP data would be better approach to really evaluate the data (Figure 1A)

For the in vitro assays, it would be nice to see that they do form G4 structures (methods described gel mobility changes) to confirm the formation

Discussion:
Spelling mistake at the end of first paragraph DAN2+/- instead of DNA2+/-

1st Revision - authors' response 25 February 2013

Point-by-point responses to the reviewers' comments:

We would like to thank the reviewers for their careful and constructive comments on our work. In the last three months, we have conducted experiments suggested by the reviewers. The new experimental results included in the revised manuscript directly address the reviewers' concerns and provide additional support to our main conclusions. Because of the additional data, we have re-arranged the figures. We have also revised the Introduction and Discussion sections according to the reviewers' suggestions. Changes in the text are specified in the point-by-point responses and indicated by vertical lines in the right margins. The following are the point-by-point responses to the reviewers' critiques:

Referee #1

Figure 1:

The ChIP is not convincing and needs repetition, quantification and error bars. Similarly The IF is of very poor quality and colicalization could be a result of random overlap, and need quantification. Along the same lines, the IPs need further standard controls, such as input, a control with another flag tagged protein that does not interact with DNA2, antibody only, beads only...
I indicated this manuscript as of low technical quality for Figure 1 only, the other figures are much better.

*We repeated the ChIP and carried out quantification (**Figure 1B**). We further knocked down mouse DNA2 and conducted ChIP on DNA2 knockdown MEF cells. We found that knockdown of DNA2 greatly decreased telomere DNA that was pulled down by the anti-DNA2 antibody (**Figure 1C-E**). In addition to the dot blotting analysis (**Figure 1C and 1D**), we also performed qPCR with telomeric primers to measure the amount of telomere DNA that was pulled down by the anti-DNA2 antibody (**Figure 1E**). The data are consistent with that of the dot blotting analysis (Page 9, lines 3-9).*

*The DNA2/telomere colocalization analysis was performed by acquiring multiple Z-stack images to avoid coincidental overlapping signals in different planes of the cell. Basically, Z-stack images were taken at approximately 0.275micron thickness per slice. In the original manuscript, images from only one stack were shown. We now provide the overlayed image from all stacks in **Figure 1F** (Extended field of all stacks) and in **Supplemental Fig. S1** (Individual stack) to show that co-localization is not due to random overlapping (Page 9, lines 10-13).*

In addition, we would like to point out that in the original submission, IF-FISH was performed using cells fixed with paraformaldehyde. In the revised version, we fixed cells with methanol/acetone and then performed IF-FISH. Methanol fixation extracts proteins that do not bind to DNA, so that the background signal is significantly reduced. Meanwhile, perhaps because methanol/acetone fixation precipitates protein, the DNA2 staining appears to be more punctuated methanol than with paraformaldehyde fixation. In addition, the stringent washing condition (70% formamide) required in FISH may also contribute to the punctuated staining, as it strips more background staining.

*The data for DNA2-telomere protein interactions is now illustrated in a new figure (**Figure 2**). New co-IP of endogenous DNA2, TRF1, and TRF2 were performed. Beads only and IgG beads were used as co-IP controls. GAPDH was used as internal loading control (**Figure 2A-2C**). We also co-expressed Flag-DNA2 and Myc-TRF1 or Flag-DNA2 and Myc-TRF2 in 293T cells. Co-IP was used to further demonstrate the interaction between DNA2 and TRF1 or TRF2. In this assay, we used Flag-GFP as a negative control (Page 9, lines 14-20).*

Figures 2, 3, 4:
The mouse deletion and telomere analysis here is far more convincing and very well executed. Similarly, the in vitro experiments are beautiful.

Figure 5:
The data presented here are convincing due to the quantification. The images are still poor quality though. Also, this figure could be combined with Fig 1.

***Figure 5 is renamed as Figure 7 due to the additional figures.** IF-FISH on H2AX in WT and DNA2+/- MEF cells was repeated. Varying antibody or telomere probe concentrations were tested in order to improve the IF-FISH quality. Images with considerably better quality and resolution are shown in **Figure 7A**. In order to clearly understand DNA2 deficiency-induced DNA damage at telomeric and non-telomeric regions, we quantified γ-H2AX-positive cells with or without telomere dysfunction induced foci (TIF). Cells with ≥ 5 γ-H2AX foci were scored as γ-H2AX-positive cells. Only the clearly and completely co-localized H2AX and telomere signals were considered as TIF. Cells with ≥ 5 foci were scored as TIF-positive cells. The new quantification and statistical analysis were summarized in **Figure 7B (**Page 14, lines 16-21).*

Because this panel compares the TIF in WT and DNA2+/- MEF cells, we decided to keep this panel in Figure 7.

Figures 6, 7:
These approaches are again highly convincing and well executed.

Referee #2

1) The colocalizations in Figure 1B are not at all convincing, though a higher resolution version of the figure might show some improvement. The image is highly pixelated, to the extent that most of the foci appear square. In addition, all the colocalizing foci only partially overlap. The DNA2 signal also appears oversaturated. Finally, colocalization is always more convincing if multiple Z-stacks are imaged, to rule out coincidental overlapping signals in different planes of the cell.

The DNA2/telomere colocalization analysis was performed by acquiring multiple Z-stack images to avoid coincidental overlapping signals in different planes of the cell. Basically, Z-stack images were taken at approximately 0.275micron thinkness per slice. In the original manuscript, images from only one stack were shown. We now provide the overlayed image from all stacks in ***Figure 1F*** *(Extended field of all stacks) and in* ***Supplemental Fig. S1*** *(Individual stack) to show that co-localization is not due to random overlapping (Page 9, lines 10-13).*

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In addition, we would like to point out that in the original submission, IF-FISH was performed using cells fixed with paraformaldehyde. In the revised version, we fixed cells with methanol/acetone and then performed IF-FISH. Methanol fixation extracts proteins that do not bind to DNA, so that the background signal is significantly reduced. Meanwhile, perhaps because methanol/acetone fixation precipitates protein, the DNA2 staining appears to be more punctuated than with paraformaldehyde fixation. In addition, the stringent washing condition (70% formamide) required in FISH may also contribute to the punctuated staining, as it strips more background staining.

2) A siRNA control was provided to verify western blot bands using the DNA2 antibody (Figure S4); similar control experiments should be provided to verify that the antibody gives specific signals in ChIP and IF experiments.

*We knocked down mouse DNA2 and conducted Western Blot, IF, ChIP on DNA2 knockdown MEF cells (****Supplementary Figure S6, Figure 1C-E****). We found that knockdown of DNA2 greatly decreased telomere DNA that was pulled down by the anti-DNA2 antibody (****Figure 1C****). These new results clearly support that mammalian DNA2 is specifically localized to telomeres (Page 9, lines 3-9).*

The siRNA control for the antibody for IF is shown in ***Figure S6****.*

3) Commercial antibodies to TRF1 are notoriously unreliable, so similar siRNA controls should be performed to verify IF signals from the TRF1 and TRF2 antibodies. In human cells in particular (Figure S1), the staining from DNA2, TRF1 and TRF2 antibodies seems to be largely cytoplasmic or perinuclear.

*We repeated the IF staining on MEF cells with or without knockdown of TFR1 or TRF2 as suggested. We also tested different blocking conditions and TRF1 or TRF2 antibodies from different companies; we were able to improve the image quality. Now, only a few non-specific IF signals from the TRF1 and TRF2 around the perinuclear area (****Figure 2F****). (Page 9, lines 21, 22)*

On the other hand, because we focus on investigating the localization of DNA2 to telomeres in MEF cells and its interaction with TRF1 and TRF2 in mouse cells, and the improved data from our ChIP, Co-IP, and IF have clearly supported this aim, we decided not to include the extra IF images in HeLa cells. However, we emphasize that DNA2 is known to be highly concentrated in the cytoplasm and functional in mitochondria, as previously reported by us (Zheng et al., 2008, Mol. Cell) and others (Duxin et al, 2009).

4) Staining for telomeric DNA in figure 5A is also poor quality, with a lot of diffuse background.

IF-FISH on H2AX in WT and DNA2+/- MEF cells was repeated. Varying antibody or telomere probe concentrations were tested in order to improve the IF-FISH quality. The image with considerably better quality and resolution were shown in ***Figure 7A****. In order to clearly understand DNA2 deficiency-induced DNA damage at telomeric and non-telomeric regions, we quantified γ-H2AX-positive cells with or without TIF. Cells with ≥ 5 γ-H2AX foci were scored as γ-H2AX-positive cells. Only the clearly and completely co-localized H2AX and telomere signals were*

considered as telomere dysfunction induced foci (TIF). Cells with ≥ 5 foci were scored as TIF-positive cells. The new quantification and statistical analysis were summarized in ***Figure 7B. (****Page 14, lines 16-21)*

5) In Figure 3A, how are sister telomere associations distinguished from chromosomes which have lost the telomeric DNA from a single chromatid?

*In our original manuscript, we followed the procedures in published articles and scored the strong single telomere FISH signal that in the middle of two associated chromosomal arms. The single FISH signal at one chromosome arm was considered as telomere loss from a single chromatid. However, we agree that FISH alone is perhaps not the best way to distinguish STA from telomere loss at one sister telomere. Only CO-FISH can clearly show sister telomere association (****now Figure 4C****). To more accurately present our results, in the revised manuscript, we have removed the STA data based on regular telomere FISH.*

6) The lower right panel of Figure 3D demonstrates that a high proportion of chromosome ends have undergone T-SCE events, but I don't understand why a similarly high proportion is not observed in the panel above, which uses the same technique.

T-SCE images shown in ***Figure 4 (previously Figure 3)*** *were selected from of CO-FISH images taken from metaphase spreads that show high frequency of T-SCE. As shown in the quantification in* ***Figure 4G****, not all metaphase spreads display T-SCE. In fact, spreads exhibiting telomere loss, fragile telomere, and sister telomere associations are less likely to have T-SCE. There is why T-SCE is not observed in other panels. At present, we do not quite understand why DNA2 deficiency induces high T-SCE. It may be relevant or irrelevant to its function in promoting telomere replication. In the future, we will explore DNA2's role in telomere recombination.*

7) In Figure 7C, how are the boundaries of individual nuclei identified?

Figure 7 is now renamed as Figure 9 due to additional figures. *The nuclei were stained with DAPI, which gave a clear edge for most of the nuclei. We have clarified this issue in the figure legend (Page 25, Lines 10-13).*

In addition, we noticed that after conversion of the file into pdf file, the image quality for this panel is particularly poor. Therefore, we have replaced the images with new ones.

A more minor comment involves the synthetic G-quadruplex substrates: in Materials and Methods, it is stated that native gel electrophoresis was performed to verify formation of the folded structure. These gels should be shown, possibly as a supplementary figure.

*Native PAGE that verifies the formation of synthetic G4 substrates was included (****Supplementary Figure S7****).*

Referee #3

1. The introduction is very well written, but lacks an introduction on G4 structures and the introduction to Dna2 switches between results from yeast and humans.

We rewrote the introduction section and included an introduction on G4 structures. We would like to point out that a recent publication shows that G4 is formed in vivo at both telomeric and non-telomeric sites preferentially in late S phase (Biffi et al. Nature Chem 2013) (Page 4, Lines 16-25 and Page 5, Lines 1-15).

We also add a paragraph in the introduction to summarize current knowledge about DNA2 in yeast and a separate paragraph on humans, particularly in nuclei in humans (Page 5, Lines 17-25 and Pages 6 and Page 7, Lines 1-20).

2. Furthermore, the connection of telomere maintenance and G4 formation is completely missing and what is written is confusing and lacks current and past important in vivo and in vitro data,

e.g. Zaugh 2005, Paeschke et al 2005, Bonetti 2009, Smith 2011, and others.

We have now discussed the connection of telomere G4 formation and telomere integrity in the Introduction section. Especially important is the new publication by Biffi et al., showing through the use of anti-G4 antibody that human telomeres likely form G4 and have functional implications, since they form in a cell cycle specific fashion requiring DNA replication (Page 4, Lines 16-25 and Page 5, Lines 1-15). Due to the limited space, we only included the most relevant references.

3. Many helicases, which contribute to genome stability at telomeres, can unwind G4 structures. In the manuscript they mention that WRN and BLM helicase unwind G4 structures in a different manner than Dna2. This is correct but also not surprising, because Dna2 cleaves G4 and WRN and BLM unwinds the structure. In addition WRN and BLM are not the only helicases that unwind G4; Pif1, FANCJ, RTEL, and other helicases have a similar unwinding potential. The current research suggests that different helicases might act at different G4s. A detailed discussion would be essential to thoroughly evaluate their Dna2 data.

Our current work focuses on a novel function of the nuclease activity of Dna2 in resolution of G4 structures. We agree with the reviewers that many helicases including WRN, BLM, FANCJ, and RTEL can potentially unwind the G4. Previous studies and our current work suggest that both the DNA2 nuclease-mediated resolution of G4 and the helicase-mediated unwinding G4 are important in maintaining telomere integrity. In the revised manuscript, we have discussed these two pathways in more detail (Page 18, Lines 1-17).

In addition, we would like to clarify that we did not mean to suggest that different helicases might act at different G4s. It is not the focus of the manuscript.

4. The interesting data about Rqh1 sumoylation and its connection to telomere replication is missing from their discussion. It would also be interesting to see, if there is a connection between Dna2 and Rqh1 during telomere replication. Sumoylation of Rqh1 is suggested to affect telomere binding and influence telomere replication allowing unwinding of G4s.

We have included Rqh1 sumoylation in the revised manuscript (Page 4, Lines 6, 7).

We agree with the reviewer that this is an interesting question to answer, but we consider that it may be beyond the scope of our current study. We will investigate whether Dna2 functionally and/or physically interacts with non-modified and sumoylated Rqh1 to promote telomere replication in the future.

5. The current manuscript completely lacks a discussion about the difference between inter and intramolecular G4. From past research it is known that intermolecular G4 and intramolecular G4 are regulated differently and are proposed to have different function. Can Dna2 cleave both structures?

It was previously shown that yeast and human DNA2 could cleave <u>both</u> inter- and intra-molecular G4 in vitro (see Masuda-Sasa et al. 2008 in the references).

What is their specific binding constant (Kd). Detailed in vitro analyses of Dna2 would be useful to evaluate their data. Also control substrates would be useful.

Dna2 binds junctions of B and non-B DNA. It was shown that human DNA2 binds both intra and intermolecular G4. The specific binding constant of DNA2 in complex with different DNA substrates was given (Masuda-Sasa et al, 2008 in references).

6. They show convincingly that Dna2 interacts with TRF1 and TRF2, but their story stops short after this very interesting finding. How is the interaction facilitated and why? Is there a consequence for telomere replication? This could be easily addressed by different in vitro assays or pull downs.

In our current study, we simply used TRF1 and TRF2 <u>as molecular markers</u> for telomeres similar to the telomere DNA probe in IF-FISH. We conducted co-IP and IF co-staining of DNA2/TRF1 and DNA2/TRF2 to demonstrate that DNA2 co-localizes to telomeres. The impact of TRF1 and TRF2 on DNA2's function will be studied in the future.

7. There are other telomere-binding proteins known that affect G4 structure like Pot1, Mre11, Cdc13, WRN and BLM, and others. How do their function fit into the current model?

We consider that the WRN- and BLM-mediated helicase-dependent G4 unwinding and DNA2 nuclease-dependent G4 cleavage are two different pathways to counteract G4. We have discussed this issue in the revised manuscript (Page 18, Lines 1-17).

On the other hand, Cdc13 inhibits Dna2 (our unpublished results), and BLM and Mre11 stimulate DNA2 nuclease activity (Nimonkar et al. 2011). The impact of Cdc13, BLM, Mre11, Pot1, TRF1, and/or TRF2 on DNA2 nuclease-mediated G4 resolution will be addressed in our future studies.

8. Dna2 has many different functions. The best-studied role is during Okazaki fragment maturation. Could it be that the observed genome stability defects are a consequence of mis-regulated Okazaki fragment maturation genome-wide. Experiments addressing this concern are important to support their hypothesis.

*We completely agree that there are additional roles for DNA2 in maintaining nuclear genome integrity. Our new finding is that there is also a telomere defect in cells lacking DNA2. In fact, we showed that DNA2+/- cells were sensitive to aphidicolin and accumulated significantly more chromosome breaks than the WT cells. This suggests a role of DNA2 in counteracting DNA replication stress at non-telomere regions. However, we should have made the roles of DNA2 in nuclei more clear. In the revised manuscript, we point out that the DNA2 foci that do not overlap with telomere foci indicate the other previously reported roles (**Figure 1F, Page 9, Lines 11-13**), and DNA2 deficiency resulted in more aphidicolin-induced chromosome breaks (**Supplementary Figure S2, Page 10, Lines 21-24 and Page 11, Lines 1-5**). We also summarize previous work published on this issue more completely in the Introduction.*

*More specifically, we and other groups have shown that DNA2 is not required for every Okazaki fragment. Instead, FEN1 is the main nuclease of Okazaki fragment processing. This has been discussed at length in many reivews. A recent study by Dr. Stewart's and Dr. Campbell's groups (Duxin et al, 2012) studies this issue directly in human cells. They detected no defects in Okazaki fragment maturation or slowing of fork progression, i.e. no genome-wide defect. Consistent with this study, we found that DNA2 deficiency did not result in obvious spontaneous chromosome breaks (< 1%, Supplementary Figure S1). To further test whether DNA2 deficiency impacted genome-wide DNA replication, we conducted additional experiments to measure the DNA incorporation rate in primary WT and DNA2+/- MEF cells (P1). We found that DNA2 deficiency had little effect on the overall efficiency of DNA replication (**Supplementary Figure S3**).*

Our current study suggests an important role of mammalian DNA2 in maintaining telomere integrity and genome stability. There may, of course, be other functions of DNA2 in maintenance of nuclear genome integrity, as DNA2 also has non-telomeric localization in nucleus (Fig. 1F). For instance, DNA2 may be important for counteracting DNA replication stress. We showed that DNA2+/- MEF cells are more sensitive to aphidicolin treatments producing chromosome breaks (Supplementary Figure S2). We have revised our manuscript and discussed the impact of both telomeric dysfunction and defects in counteracting replication stresses on genome instabilities and cancer development (Page 18, Lines 19-25, Page 19 Lines 1-14).

9. Dna2 is also essential in the mitochondria. The major protein population of Dna2 is in the mitochondria. It is well known that mitochondria defects contribute greatly to the overall phenotype of the cell. To address this question it would be good to analyze the mitochondrial defect in detail in order to compare it to the nuclear function.

Previously, we demonstrated that the majority of DNA2 migrated into mitochondria (Zheng et. al Molecular Cell 2008). In a very recent study, we identified DNA2 mutations in human mitochondrial myopathy patients and showed these DNA2 mutations impaired its biochemical activity and caused mitochondrial defects (Ronchi et.al Am J Hum Genet 2013). The paper was published during the revision of our current manuscript. We have discussed this work in the Introduction. On the other hand, our current work focuses on a fundamental unanswered question: what is the function of the subset of DNA2 that is translocated into nuclei? Here, we demonstrate that DNA2 plays a critical

role in telomere stability and DNA2 deficiency causes telomere dysfunction. We consider that there may be more functions of DNA2. In the future, we will reveal those functions and address the relationships among different DNA2 functions in nuclei and mitochondria.

With respect to the amount of Dna2 in the nucleus, we also note in the Introduction, that there appears to be as much Dna2 in nuclei as there is pol delta and several other replication initiation proteins as judged by co-immunoprecipitation instead of by IF (Duxin et al., 2012; Peng et al., 2012).

10. G-quadruplexes are a very important and currently highly discussed topic. Many scientists are trying to address the question if G-quadruplex motifs can form G-quadruplex structures in vivo and if so, what their function might be. It would be beneficial for the reader to know what the function of G4 structures are at telomeres and if the authors think that G4 form always at telomeres.

We have added a paragraph in the introduction to discuss the formation of G4 structures in vivo (Biffi et al, 2013; Paeschke et al, 2011; Paeschke et al, 2005; Smith et al, 2011). The formation of G quadruplexes in vivo has recently been demonstrated with antibodies and cytology (Biffi et al. Nature Chem 2013) (Page 4, Lines 16-25 and Page 5 Lines 1-15).

11. TMPyP4 is not an ideal G4 stabilization ligand. TMPyP4 is not specific for telomeres, it is also used in general to stabilize G4 structure. Furthermore recent publications show that it can also bind and stabilize duplex DNA. The observed effect could also be that many G4 structures (in human over 300 000) are stabilized and effect replication events or that the stabilized duplex DNA acts differently during DNA replication. G4 ligands are a nice addition for some analyses, but alone not proof of G4 formation. The ligand can even induce the formation of such structures. A more critical evaluation here is essential.

*We know of no absolutely telomere specific G4 stabilizer. We are presenting a correlation between telomere dysfunction in the Dna2 defective cells and increased G4 formation or presence at telomeres. Other sites could be involved but we haven't investigated them. To show that the G4-stabilizer-induced fragile telomere phenotype was due to G4 formation, we included another G4 stabilization molecule telomestatin, which was previously demonstrated to stabilize G4 at telomeres. Our new data clearly show that increased G4 formation or presence caused more fragile telomeres in DNA2+/- MEF cells than in the WT cells (**Figure 6**). It suggests that DNA2 nuclease removes G4 structures to reduce replication stress at telomeres and thereby preserve telomere integrity.*

Minor comment

Introduction:
Several Refs. are cited wrong or are missing: e.g. in the introduction a REF (no review) showing G4 dependent block of telomere replication would be useful. The later cited articles Sfeir 2009 do not show this, they only argue in one sentence that G4 structure might help to understand their data, Bochman et al. is a review article and Vannier et al. is also not showing direct evidence that G4 block DNA replication at telomeres.

*Two original research articles that show formation of G4 structures block DNA replication **in vitro** are cited. In addition, Paeshcke et al. 2011 is now cited, in which the Zakian lab shows directly, in yeast, that G-rich sequences block replication forks, though not explicitly at telomeres. Several citations that suggest replication forks are blocked at telomeres are also cited, though they did not explicitly study G4s. The cited review is the most comprehensive discussion of this point.*

One of the last sentences on Page 4 has grammar issues: " ....how do nucleases/helicases resolve telomeric G4, is G4 unwindig is the only way....." is not correct.

We have made the correction.

G4 is used before the G-quadruplex (G4) is introduced.

We have made the correction.

Results:
qPCR analysis of their ChIP data would be better approach to really evaluate the data (Figure 1A)

qPCR has now been carried out and data are included (Figure 1D).

For the in vitro assays, it would be nice to see that they do form G4 structures (methods described gel mobility changes) to confirm the formation.

Native PAGE on G4 and control model substrates is included (Supplementary Figure S6).

Discussion:
Spelling mistake at the end of first paragraph DAN2+/- instead of DNA2+/-

We have corrected the error.

2nd Editorial Decision 13 March 2013

Thank you for submitting your revised manuscript for our consideration. Two of the original reviewers have now reviewed it once more (see comments below), and I am pleased to inform you that they have no more principle objections towards publication in The EMBO Journal. We shall therefore be happy to accept your study, once the following remaining editorial issues have been satisfactorily addressed:

- please correct the errors pointed out by referee 2, and carefully proofread the text for any other errors of such kind

- when correcting the title, I would also propose a slight rewording to make it more attractive and accessible, focussing on the key novel aspects and expressing them clearly - suggestion: "Mammalian DNA2 helicase/nuclease cleaves G-quadruplex DNA and is required for telomere integrity"

- please carefully revise both the main and the supplementary figures regarding image quality. I noticed that various inserts are of low resolution/too pixelated (see e.g. some of the DNA model drawings in Figure 5B-D). Moreover, many of the gel and blot panels and their relation to the original data are difficult to assess because of low image quality and/or contrast/brightness adjustments that lead to loss of background signals (see just for some examples Fig. 5C-D or Fig 3E). Therefore please revisit all such panels in the main manuscript and the supplement, revising them carefully to ensure accurate representation of the original data.

- finally, in order to complement the required image improvement efforts, please also provide source data files for the various electrophoretic gels and blots in this case. We would ask for a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

I am therefore returning the manuscript to you for one final round of minor revision, hoping you will be able to upload and re-submit the final corrected version as soon as possible. Should you have any questions in this regard, please do not hesitate to contact me directly.

REFEREE COMMENTS

Referee #1

The authors have addressed my concerns very well and I am satisfied with the revised version.

Referee #2

The authors have addressed all of my concerns adequately. The new data are of much higher technical quality than the original images, and I am satisfied that the data support the conclusion that DNA2 localises to mouse telomeres and plays a role in telomere stability.

I noticed some textual errors that should be corrected prior to publication:
1) "G-quadruplex" is spelled incorrectly in the title, the abstract, and at the bottom of page 3.
2) The statement on p4: "G4 structures form in a cell cycle specific manner and are absent in S phase in both organisms...." is incorrect; in human cells there is an INCREASE in G4 antibody signals in S phase (Biffi et al 2013).
3) p5, line 12: "complimentary" should be "complementary"
4) p7, line 23: In the sentence "To determine if human DNA2, like yeast DNA2, is important in telomere DNA metabolism...", I think the authors mean mammalian DNA2, not human, since the rest of the sentence refers to mouse cells. Human and mouse telomere biology are very different, so one cannot assume a priori that a particular protein will have the same telomeric function in both species.

2nd Revision - authors' response 25 March 2013

Responses to the reviewers' comments

We would like to thank the reviewers for their tremendous efforts in reviewing our work (EMBOJ-2012-83769R). We are happy to know that they consider our additional data are sufficient to address all of their concerns. We have made all corrections following the additional comments from referee 2.

1) "G-quadruplex" is spelled incorrectly in the title, the abstract, and at the bottom of page 3.

We have made corrections.

2) The statement on p4: "G4 structures form in a cell cycle specific manner and are absent in S phase in both organisms...." is incorrect; in human cells there is an INCREASE in G4 antibody signals in S phase (Biffi et al 2013).

We have corrected the error.

3) p5, line 12: "complimentary" should be "complementary"

We have corrected the error.

4) p7, line 23: In the sentence "To determine if human DNA2, like yeast DNA2, is important in telomere DNA metabolism...", I think the authors mean mammalian DNA2, not human, since the rest of the sentence refers to mouse cells. Human and mouse telomere biology are very different, so one cannot assume a priori that a particular protein will have the same telomeric function in both species.

We agree with the referee and made the change as suggested.

