FANCJ promotes replication of G-quadruplex DNA structures

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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.)

Pre-Decision 05 May 2014

Thank you again for submitting your manuscript EMBOJ-2014-88663, "FANCJ promotes replication of G-quadruplex DNA structures." We have now received all the reports from three experts, which I am enclosing copied below. As you will see, the reviewers express some interest in the basic finding of your study, yet they also raise a number of substantive concerns, and issues with the potential to confound key conclusions of the study. Before taking a final decision, I would in this light be interested to hear your response to these comments and how you could imagine addressing the main conceptual problems, in particular those raised by referee 3. I am therefore giving you an opportunity to consider the enclosed reports amnd discuss them with your coworkers, and to contact me with a tentative response letter detailing whether and how you might be able to address the concerns if offered the possibility to revise the manuscript. These tentative response (parts of which we may choose to share and discuss with some of the referees) would be taken into account when making our final decision on this manuscript. I would therefore appreciate if you could send us such a response at your earliest convenience, ideally by early next week.

Referee #1

In this paper Bosch et al., describe various studies on DNA replication using DNA substrates with and without guanine-rich DNA capable of forming guanine quadruplex structures. A phage ssDNA model as well as a model system based on Xenopus egg extracts was developed to study replication of G-rich DNA in eukaryotic cells. With both systems it is shown that G-quadruplex structures form
a barrier for DNA replication. With the Xenopus system it is shown that nascent strand synthesis is blocked one or two nucleotides from the predicted G4 structures in line with previous studies in C.elegans lacking dog-1/FancJ. Persistent replication stalling at G-quadruplex structures was observed following depletion of the FANCJ/BRIP1 helicase in Xenopus extracts, pointing to an important role for this helicase in resolving G4 structures.

This is an excellent study that adds significantly to the literature on G4 structures and the accumulating data indicating that genomic instability can result from failure to resolve these structures during replication, transcription and/or recombination. I only have a few minor points of criticism.

1. The exclusive focus on intramolecular G4 structures seems unfounded. Perhaps even in the phage assay multiple single stranded DNA substrates could come together to form intermolecular G4 structures.

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3. The role of epigenetic marks in replication of G-rich DNA and the role of various ions such as K+ relative to what is likely to be present in the nucleus should be discussed. Limitation of studies with purified ssDNA relative to the more complex chromatin environment in a cell should also be discussed.

Referee #2

The authors examined the function and connection of G-quadruplex structures (G4) and replication. In this study they used a cell free system based on Xenopus egg extracts to investigate G4 decomposition and replication. Their particular focus was the effect of G4 DNA on replication stalling. In their study they report that G4 structures have an intrinsic capability to block DNA replication. In addition they showed that the helicase FANCJ is essential for efficient DNA replication past these G4 sites. Their data provide evidence that the replication machinery stops in front of the G4 site prior to G4 unwinding. This unwinding allows DNA replication to pass these obstacles and consequently genome stability is preserved.

Issues of concern:

Major points

1. In Figure 1 they introduce nicely four different controls. These controls are chosen very wisely because they reflect various situations. Nevertheless, in the whole paper only one control is stated and depicted. Especially the G2N is essential and very informative to show in the paper, because G2N can in theory also form into G4 structures, although very weak.

2. It is well known that a sequence that contains a G4 motif does not consequently form into G4 structures. CD analyses of G4s and control-sequences are essential to strengthen their data and arguments. In addition it would be very informative to see CD spectra of the plasmid with and without G4s.

3. It would be beneficial to show error bars in Figure 2C and as stated above to see all more controls.

4. Additional information would be obtained, if Phen-DC would be included in Figure 2.
5. A major problem of the presented data is that all analyses are done with G4 on the plasmid. Due to the supercoiled nature of the plasmid it is not clear, if G4 structures form and behave the same way on a plasmid as in a single-stranded conformation. Therefore it would be essential to confirm the observed effect with a single-stranded oligonucleotide or linearized plasmids to eliminate the potential that all observed effects are only due to G4 motifs on the plasmid.

6. Phen-DC has so far only been tested in S. cerevisiae. It is not clear how the ligand acts in a cell free system or in Xenopus. In general it is not clear, if ligands induce or stabilize G4 structures. Therefore, using an alternative ligand such as TMPyP4 would be essential to strengthen their conclusions. In addition, an appropriate and essential control for all presented ligand data would be the use of TMPyP2, which has a similar chemical structure as TMPyP4 but can not induce or stabilize G4 structures.

7. Figure 3 is hard to understand. The authors argue that the differences they observe might be due to different G4 structures. This needs further explanation and experimental evidence by testing more G4 motifs with similar loop size or similar G- tracts. This observation, also highly relevant, is not discussed in the discussion. It would be very interesting to see if the stability (maybe melting curves can support this observation) or the structure (parallel, antiparallel) of a given G4 is the cause of this difference. An alternative idea would be to test if different monovalent cations (KCl results in strong G4 whereas LiCl in weaker) alter the observed phenomena.

8. On page 12, lane 10 it is not clear what G4G5N is referring too. Therefore, the whole paragraph is not clear and is rather confusing.

9. Figure 4 nicely illustrates the effect of Phen-DC on G4-dependent replication stalling. It is essential to also show (same conditions as C,D) DNA replication of control samples (non G4) in the presence of Phen-DC.

10. The obtained results are very interesting and clear. To further validate the function of FANCJ and G4 structures and their role during DNA replication it would be essential to check DNA replication at G4 substrates also in the absence of other human helicases such as BLM or WRN. These experiments would strengthen the hypothesis that FANCJ is required for G4 unwinding prior DNA replication and not just simply any given helicase.

Minor points:

1. In the abstract the authors state that G4 structures play a role; this is misleading, because to this date it is a hypothesis that G4 structures could be involved in biological processes such as DNA replication, transcription... 

2. In the abstract, lane 7, is a spelling mistake: from instead of form.

3. In the whole manuscript the authors switch between G4 and G-quadruplex

4. It is very confusing for a non G4 person to understand the differences of G4 structures, motifs, sequences, DNA or substrate. A consistent nomenclature, which is introduced and explained, will be beneficial.

5. In the introduction it will be helpful to rearrange the Figure panels. It will be helpful to cite Figure 1B already in lane 8 when the motif is first introduced.

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9. In the result section the author switch tense between past and present. It would be beneficial to stay in present (e.g. page 14)

10. Total gels of Figure 4 C, D will help to better understand the data in the whole context.

11. Figure 5D, why is the bottom gel for the Mock sample only 2-40 minutes visualized?

12. On figure 5D in both bottom gels a very short band appears in the first lane (2 min)- is this observed in all assays and only visible here due to a different gel processing? What is the nature of this band?

Referee #3

In this manuscript, the authors investigate the effects of G quadruplexes (G4) on DNA replication using a nice in-vitro system that allows the replication of single-stranded DNA. They show that DNA synthesis transiently stalls at G4 and suggest that FANC J is involved in the resolution of these structures. This is a technically interesting piece of work, that provides useful information but the most important conclusions are clearly overstated and could be misleading. More precise comments that could improve this manuscript are below.

1. A major and important criticism concerns the role of FANC J in the resolution of G4 structures during DNA synthesis. The authors state several times that G4 structures are not resolved in the absence of FANC J (see for example page 13, first paragraph). However, all the figures show that up to 90% of these structures are resolved relative to the control. This is emphasized in the comparison of Figure 5B (middle panel) and Figure 5D (lower right panel). Both panels concern the same experiment, one showing that a maximum of 10 % of G4 remained unresolved and the other showing that nearly all G4 were resolved. The statement that FANC J cause persistent stalling at G4 (also written in the summary) is therefore not in agreement with the data shown in the manuscript. In my opinion, this manuscript shows only a mild effect of FANC J depletion and it is difficult to escape from the conclusion that most G4 are also resolved in the absence of FANC J. The title of the manuscript is also misleading.

2. Based on Figure 3 data, it is difficult to state in the text and figure legend that the stalled band is at 760 nt from the G4 as the shown gels did not allow a good resolution. Moreover, no molecular weight makers are shown. Figure 4 gives the correct information, but the presentation of the results should be reformulated to avoid premature conclusions that are not in agreement with the shown data.

Other comments:

. Figure 4B should indicate in the ordinate the size of some of the corresponding bands in the sequencing ladder.

. Page 12: and corresponding Figure 4D. I guess that the gel corresponds to G4G23, and not G4G3N. In addition, the text refers to G4G5N. This part should be checked.

. Figure 7 does not bring any new information.
Thank you for sending the reviewer reports. We are glad that all reviewers find our work of interest. We have looked carefully at their response and we can address most issues that were raised. I will address the concern raised by reviewer #3 in the next paragraphs and I will attach a file with some extra information and our response to other reviewer comments.

The main issue raised by reviewer #3 concerns our claim that FANCJ plays an important role in replication of G-quadruplex DNA. He/she points out that after FANCJ depletion we only observe persistent replication stalling in ~10% of the G4 substrates and that stalling does not persist equally long in all experiments. We would like to clarify two important aspects of our data. First, on a technical note, the effect of FANCJ depletion on the duration of replication stalling depends on the efficiency of depletion. To clarify this we have added an experiment to the attached file showing that more than 2 consecutive rounds of FANCJ depletion are required to see fully persistent replication stalling. In retrospect, we may not have chosen the best experiment for Figure 5D, it was one of our earlier experiments in which we used only 2 rounds of depletion. While it served our aim to show the effect of PhenDC3 on replication stalling, it was not the best FANCJ depletion. We can easily repeat this experiment using more extensive FANCJ depletion conditions to show that replication stalling is persistent.

This brings us to the raised concern that not all G4 structures in a given population seem to be substrates for FANCJ. We totally agree that there are likely other mechanisms to resolve G4 structures during DNA replication, in addition to the FANCJ-dependent mechanism we here describe. We clearly address these in the discussion, and in Figure 7, hence it surprised us that reviewer #3 used the word ‘misleading’. We however will adjust some of the phrasing to be more clear on this issue. We do not claim that there are no alternative ways to deal with structural replication fork impediments, and many additional experiments will be required to identify and characterize all of these. But, our genome contains more than 300,000 potential G4 sites, even if only a small fraction of those form a G-quadruplex at a given time, there would still be a considerable number of G4 structures causing problems in the absence of FANCJ.

To us, our paper clearly and convincingly demonstrates that G4 structures block DNA replication and that FANCJ promotes replication across a significant fraction of them. Reviewer #1 and #2 seem to agree and are very positive about our work. We feel that a few of the experiments suggested by reviewer #2, although by themselves interesting, really go beyond the scope of the paper and are also not central to our study. This specifically concerns a structure-function analysis of G-quadruplex forming sequences and the testing of numerous other helicases. We will experimentally address all other comments but hope that you will understand that the above mentioned experiments are too time consuming and will cause a considerable delay in publishing our competitive data.

We greatly appreciate you giving us the opportunity to address these issues and look forward to hearing your response.
Referee #1

(Report for Author)

In this paper Bosch et al., describe various studies on DNA replication using DNA substrates with and without guanine-rich DNA capable of forming guanine quadruplex structures. A phage ssDNA model as well as a model system based on Xenopus egg extracts was developed to study replication of G-rich DNA in eukaryotic cells. With both systems it is shown that G-quadruplex structures form a barrier for DNA replication. With the Xenopus system it is shown that nascent strand synthesis is blocked one or two nucleotides from the predicted G4 structures in line with previous studies in C.elegans lacking dog-1/FancJ. Persistent replication stalling at G-quadruplex structures was observed following depletion of the FANCI/BRIP1 helicase in Xenopus extracts, pointing to an important role for this helicase in resolving G4 structures.

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1. The exclusive focus on intramolecular G4 structures seems unfounded. Perhaps even in the phage assay multiple single stranded DNA substrates could come together to form intermolecular G4 structures.

   We currently have no indications that intermolecular G4 structures form. Our substrates are used at a low concentration and do not change mobility on native gels after induction of the G-quadruplexes. However, we can not exclude the presence of intermolecular G4 structures and we will address this in the manuscript.

2. The characterization of the FancJ antibody used for depletion of Xenopus extracts is minimal. Can it be excluded that this antibody also binds to other G4 resolving enzymes such as XPD, RTEL1, WRN and BLM? The results now suggest that FANCJ is the only enzyme required to unwind the G4 structures used in this assay. Are the other enzymes mentioned not relevant or not present in Xenopus egg extracts? Results with the Xenopus system using other substrates with G4 potential such as e.g. telomeric DNA would increase the relevance of the paper.

Recombinant FANCJ rescues the depletion with the FANCJ antibody demonstrating that it is only the lack of FANCJ that causes the persistency of replication blocks. Although we show that FANCJ is essential to resolve a subset of G4 structures, additional mechanisms are likely in place to resolve the remaining G-quadruplexes. These could involve the suggested helicases of which definitely three (XPD, WRN, BLM), and likely all four are present in Xenopus oocytes. This will be mentioned in the text of the manuscript. Concerning the telomeric repeats, we found these not to induce G4-induced instability in dog-1 deficient C. elegans, in sharp contrast to the substrates used here. If necessary we could generate these plasmids and test them, but expect very little and will thus just lead to a delay in publication.

3. The role of epigenetic marks in replication of G-rich DNA and the role of various ions such as K+ relative to what is likely to be present in the nucleus should be discussed. Limitation of studies with purified ssDNA relative to the more complex chromatin environment in a cell should also be discussed.
We will add these subjects to the discussion.

Referee #2

(Report for Author)

FANCJ promotes replication of G-quadruplex DNA structures

The authors examined the function and connection of G-quadruplex structures (G4) and replication. In this study they used a cell free system based on Xenopus egg extracts to investigate G4 decomposition and replication. Their particular focus was the effect of G4 DNA on replication stalling. In their study they report that G4 structures have an intrinsic capability to block DNA replication. In addition they showed that the helicase FANCJ is essential for efficient DNA replication past these G4 sites. Their data provide evidence that the replication machinery stops in front of the G4 site prior to G4 unwinding. This unwinding allows DNA replication to pass these obstacles and consequently genome stability is preserved.

Issues of concern:

Major points
1. In Figure 1 they introduce nicely four different controls. These controls are chosen very wisely because they reflect various situations. Nevertheless, in the whole paper only one control is stated and depicted. Especially the G2N is essential and very informative to show in the paper, because G2N can in theory also form into G4 structures, although very weak.

We will add experiments using these controls.

2. It is well known that a sequence that contains a G4 motif does not consequently form into G4 structures. CD analyses of G4s and control-sequences are essential to strengthen their data and arguments. In addition it would be very informative to see CD spectra of the plasmid with and without G4s.

We show a G4 motif dependent block of primer extension in a potassium-dependent fashion (Figure 2 and Supplementary Figure S1), strongly arguing for the presence of a G-quadruplex. This notion is further supported by our result that sequences similar in sequence context but without the ability to form a G-quadruplex, do not block replication. To us, additional and time-consuming CD analysis of oligo’s containing our G4 sequences, although doable, have only limited value. This will still be different from a G4-containing plasmid and the buffer conditions may not be extrapolated to Xenopus egg extract. We contacted experts who told us that CD of a ~20 nt G4 structure in a ~3000 nt plasmid will not show the characteristic G4 spectrum due to high background of non-G4 DNA.

3. It would be beneficial to show error bars in Figure 2C and as stated above to see all more controls.

This will be done

4. Additional information would be obtained, if Phen-DC would be included in Figure 2.
This experiment will be done.

5. A major problem of the presented data is that all analyses are done with G4 on the plasmid. Due to the supercoiled nature of the plasmid it is not clear, if G4 structures form and behave the same way on a plasmid as in a single-stranded conformation. Therefore it would be essential to confirm the observed effect with a single-stranded oligonucleotide or linearized plasmids to eliminate the potential that all observed effects are only due to G4 motifs on the plasmid.

Single stranded DNA plasmids are not supercoiled and therefore we do not think the circular nature of our substrates is a problem.

6. Phen-DC has so far only been tested in S. cerevisiae. It is not clear how the ligand acts in a cell free system or in Xenopus. In general it is not clear, if ligands induce or stabilize G4 structures. Therefore, using an alternative ligand such as TMPyP4 would be essential to strengthen their conclusions. In addition, an appropriate and essential control for all presented ligand data would be the use of TMPyP2, which has a similar chemical structure as TMPyP4 but can not induce or stabilize G4 structures.

It is not clear to us how this would help to resolve the issue “if ligands induce or stabilize G4 structures”. We show that PhenDC3 definitely stabilizes G4 structures as leads to more persistent replication blocks (Figure 3 and 4). Whether this compound not only stabilizes but also induces G-quadruplexes will likely not be solved by adding an additional compound. However, if necessary we can test TMPyP4 and TMPyP2 in our assay.

7. Figure 3 is hard to understand. The authors argue that the differences they observe might be due to different G4 structures. This needs further explanation and experimental evidence by testing more G4 motifs with similar loop size or similar G-tracts. This observation, also highly relevant, is not discussed in the discussion. It would be very interesting to see if the stability (maybe melting curves can support this observation) or the structure (parallel, antiparallel) of a given G4 is the cause of this difference. An alternative idea would be to test if different monovalent cations (KCl results in strong G4 whereas LiCl in weaker) alter the observed phenomena.

It would obviously be very interesting to gain insight into the role of the G4 type and loop size in replication stalling using our system to match the dozens of biophysical papers that have been published over the last decade. However, doing this properly requires the generation and characterization of a large set of new substrates, and is not adding to the findings and conclusions of this study. To us this is beyond the scope of this manuscript. Instead we will discuss the outcomes of figure 3 more nuanced.

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10. The obtained results are very interesting and clear. To further validate the function of FANCJ and G4 structures and their role during DNA replication it would be essential to check DNA replication at G4 substrates also in the absence of other human helicases such as BLM or WRN. These experiments would strengthen the hypothesis that FANCJ is required for G4 unwinding prior DNA replication and not just simply any given helicase.

We have clearly shown that FANCJ is specifically required for a subset of G4 substrates since replication of these structures can only be rescued by the addition of FANCJ. Generation and validation of new antibodies to several other types of helicases (BLM, WRN, RTEL, XPD), although very interesting, go beyond the scope of our study.

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In this manuscript, the authors investigate the effects of G quadruplexes (G4) on DNA replication using a nice in-vitro system that allows the replication of single-stranded DNA. They show that DNA synthesis transiently stalls at G4 and suggest that FANC J is involved in the resolution of these structures. This is a technically interesting piece of work, that
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This reviewer points out that after FANCJ depletion we only observe persistent replication stalling in ~10% of the G4 substrates and that stalling does not persist equally long in all experiments. We would like to clarify two important aspects of our data. First, on a technical note, the effect of FANCJ depletion on the duration of replication stalling depends on the efficiency of depletion. To clarify this we have added an experiment under here showing that more than 2 consecutive rounds of FANCJ depletion are required to see fully persistent replication stalling.

\[
\begin{array}{|c|c|c|c|c|c|c|c|}
\hline
& \text{Mock} & 2x \text{AFANCJ} & 3x \text{AFANCJ} & 4x \text{AFANCJ} \\
\hline
\text{Time (min)} & 4 & 15 & 30 & 70 & 120 & 4 & 15 & 30 & 70 & 120 & 4 & 15 & 30 & 70 & 120 \\
\hline
\end{array}
\]

Figure legend: Xenopus egg extract was mock-depleted, or FANCJ-depleted for 2, 3, 4, or 5 rounds. Depleted extracts were used to replicate the G4\textsuperscript{G3N} template. Replication products were extracted, separated on 6 % urea-PAGE gels and visualized by autoradiography (top). Depleted extracts were analyzed by Western blot using a FANCJ antibody (bottom).
We show in this experiment that the degree of stalling is in agreement with the degree of depletion. After 2 rounds of FANCJ depletions there is some protein left (see Western blot) and the stalled replication product does not persist (autoradiograph). However, after 3 or 4 rounds of deletion the levels of FANCJ are under the detection limit of the antibody and the stalled band stably persists until the latest time point.

In retrospect, we may not have chosen the best experiment for Figure 5D, it was one of our earlier experiments in which we used only 2 rounds of depletion. While it served our aim to show the effect of PhenDC3 on replication stalling, it was not the best FANCJ depletion. We can easily repeat this experiment using more extensive FANCJ depletion conditions to show that replication stalling is persistent.

This brings us to the raised concern that not all G4 structures in a given population seem to be substrates for FANCJ. We totally agree that there are likely other mechanisms to resolve G4 structures during DNA replication, in addition to the FANCJ-dependent mechanism we here describe. We clearly address these in the discussion, and in Figure 7 and did not mean to be misleading. We however will adjust some of the phrasing to be more clear on this issue. We do not claim that there are no alternative ways to deal with structural replication fork impediments, and many additional experiments will be required to identify and characterize all of these. But, our genome contains more than 300.000 potential G4 sites, even if only a small fraction of those form a G-quadruplex at a given time, there would still be a considerable number of G4 structures causing problems in the absence of FANCJ. Therefore we consider this FANCJ-dependent mechanism highly relevant.

2. Based on Figure 3 data, it is difficult to state in the text and figure legend that the stalled band is at 760 nt from the G4 as the shown gels did not allow a good resolution. Moreover, no molecular weight makers are shown. Figure 4 gives the correct information, but the presentation of the results should be reformulated to avoid premature conclusions that are not in agreement with the shown data.

These are good suggestions and we will adjust accordingly

Other comments:

. Figure 4B should indicate in the ordinate the size of some of the corresponding bands in the sequencing ladder.

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. Figure 7 does not bring any new information.

We will address all these points
Thank you for response to the comments of our three referees on your recent submission, EMBOJ-2014-88663. I have now had a chance to consider your plans for revision, and I am glad to see that you may be in a good position to answer the main concerns with further clarifications and with additional data. We shall therefore in principle be able to consider an accordingly revised version of the manuscript further for publication.

For revising the study, I agree that extension to experimental study of telomeric G4 sequences or biophysical analyses of G quadruplexes are unlikely to add important insight to the present paper and would therefore not be required at this stage. Regarding the testing of other helicases and of G4 structure/function analyses in your experimental system, I appreciate the referees' point that such data would represent a valuable application of your new system, but also understand that especially the generation of helicase-depleting antibodies would require significant further work, and will therefore not insist on the inclusion of such data at the present stage. As proposed, the remainder of the issues (including the testing of alternative G4 ligands) should be addressed experimentally. Please also make sure to carefully edit and proofread the manuscript prior to resubmission, in particular with regard to writing, avoidance of jargon, and completeness of the citation information in the reference list.

I would therefore like to invite you to prepare and submit a new version of the manuscript along the proposed lines, together with a comprehensive response letter (will form part of the Review Process File, and will therefore be available online to the community), preferentially within our standard revision period of three months. As per our EMBO Journal editorial policy, competing manuscripts published elsewhere 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 for the opportunity to consider your work for publication. I look forward to your revision.

We would like to thank all the reviewers for their constructive feedback and we are pleased to address their comments below.

Referee #1

In this paper Bosch et al., describe various studies on DNA replication using DNA substrates with and without guanine-rich DNA capable of forming guanine quadruplex structures. A phage ssDNA model as well as a model system based on Xenopus egg extracts was developed to study replication of G-rich DNA in eukaryotic cells. With both systems it is shown that G-quadruplex structures form a barrier for DNA replication. With the Xenopus system it is shown that nascent strand synthesis is blocked one or two nucleotides from the predicted G4 structures in line with previous studies in C.elegans lacking dog-1/FancJ. Persistent replication stalling at G-quadruplex structures was observed following depletion of the FANCJ/BRIP1 helicase in Xenopus extracts, pointing to an important role for this helicase in resolving G4 structures.

This is an excellent study that adds significantly to the literature on G4 structures and the accumulating data indicating that genomic instability can result from failure to resolve these structures during replication, transcription and/or recombination.
I only have a few minor points of criticism.

1. The exclusive focus on intramolecular G4 structures seems unfounded. Perhaps even in the phage assay multiple single stranded DNA substrates could come together to form intermolecular G4 structures.

We currently have no indications that intermolecular G-quadruplex structures form in our plasmids. We use a very low concentration which favors intramolecular G-quadruplex formation. In addition, the control sequence that could in principle form intermolecular G-quadruplex structures (non-G4C3P) apparently does not do so since we did not observe stalling in the T7 primer extension assay (Figure 2B). We have now addressed this issue in the manuscript on p. 8, first paragraph.

2. The characterization of the FancJ antibody used for depletion of Xenopus extracts is minimal. Can it be excluded that this antibody also binds to other G4 resolving enzymes such as XPD, RTEL1, WRN and BLM? The results now suggest that FANCJ is the only enzyme required to unwind the G4 structures used in this assay. Are the other enzymes mentioned not relevant or not present in Xenopus egg extracts?

Recombinant FANCJ rescues the depletion with the FANCJ antibody demonstrating that it is the lack of FANCJ specifically that causes the persistency of replication blocks. Although we show that FANCJ is essential to resolve a subset of G-quadruplex structures, additional FANCJ independent mechanisms are likely in place to resolve the remaining G-quadruplexes (see Discussion and Figure 7D). These could involve the suggested helicases of which definitely three (XPD, WRN, BLM), and likely all four are present in Xenopus oocytes. We have mentioned these helicases in the discussion on page 16, first paragraph.

Results with the Xenopus system using other substrates with G4 potential such as e.g. telomeric DNA would increase the relevance of the paper.

Concerning the telomeric repeats, we found these not to induce G4-induced instability in dog-1 deficient C. elegans, in sharp contrast to the substrates used here. In addition, we do not mimic a telomeric replication context in our assay, therefore it would be difficult to translate any outcome to the replication of telomeric G4 sequences in vivo.

3. The role of epigenetic marks in replication of G-rich DNA and the role of various ions such as K+ relative to what is likely to be present in the nucleus should be discussed. Limitation of studies with purified ssDNA relative to the more complex chromatin environment in a cell should also be discussed.

These are all relevant points and we have addressed them in the Results or Discussion sections.

Referee #2

FANCJ promotes replication of G-quadruplex DNA structures

The authors examined the function and connection of G-quadruplex structures (G4) and replication. In this study they used a cell free system based on Xenopus egg extracts to investigate G4 decomposition and replication. Their particular focus was the effect of G4 DNA on replication stalling. In their study they report that G4 structures have an intrinsic capability to block DNA replication. In addition they showed that the helicase FANCJ is essential for efficient DNA replication past these G4 sites. Their data provide evidence that the replication machinery stops in front of the G4 site prior to G4 unwinding. This unwinding allows DNA replication to pass these obstacles and consequently genome stability is preserved.
Issues of concern:

Major points
1. In Figure 1 they introduce nicely four different controls. These controls are chosen very wisely because they reflect various situations. Nevertheless, in the whole paper only one control is stated and depicted. Especially the G2N is essential and very informative to show in the paper, because G2N can in theory also form into G4 structures, although very weak.

We have added several experiments using additional control sequences. Specifically, for the T7 primer extension assay we used control sequences C3P, A20 and G2N (A20 and G2N have been added and are shown in Supplementary Figure 1), for Xenopus egg extract replication assay we used control sequences C3P, C23 and C5P (C23 and C5P are added and shown Supplementary Figure 7B and D).

Concerning the G2N plasmid, we performed the T7 primer extension assay using this template and found that it did not block DNA synthesis indicating that it does not form a stable G-quadruplex structure in our experimental conditions (Supplementary Figure S1B). Interestingly, upon addition of Phen-DC3 we did observe some stalled extension products suggesting unstable G-quadruplex structures are stabilized, or the compound induces G-quadruplex structures in this plasmid (Supplementary Figure S4A).

2. It is well known that a sequence that contains a G4 motif does not consequently form into G4 structures. CD analyses of G4s and control sequences are essential to strengthen their data and arguments. In addition it would be very informative to see CD spectra of the plasmid with and without G4s.

We show a G4 sequence- and potassium-dependent block of primer extension (Figure 2 and Supplementary Figure S1E and S1F), strongly arguing for the presence of a G-quadruplex structure. This notion is further supported by our result that G-rich sequences similar in sequence context but without the ability to form a G-quadruplex, do not block replication. To us, additional CD analysis of oligos containing our G4 sequences are of limited value: it will still be different from a G4-containing plasmid and also the buffer conditions may not be extrapolated to Xenopus egg extract. Moreover, several experts told us that CD of a ~20 nt G4 structure in a ~3000 nt plasmid will not show the characteristic G4 spectrum due to high background of non-G4 DNA.

3. It would be beneficial to show error bars in Figure 2C and as stated above to see all more controls.

To show that the stalling of primer extension at the G4 sequence is very reproducible we have added an additional experiment to the supplement (Supplementary Figure S1C and D). We have also added additional control sequences for the T7 primer extension assay (see above).

4. Additional information would be obtained, if Phen-DC would be included in Figure 2.

We have performed this experiment and showed that in presence of Phen-DC3 primer extension is blocked at the G4 sequence. The compound also induces extensive stalling at several other sites which may suggest that it can also stabilize other secondary structures (Supplementary Figure S4A). Importantly, however, no G4-independent stalling bands were observed in the Xenopus egg extract assay (Figure 3D and Supplementary Figures S3B, S6).

5. A major problem of the presented data is that all analyses are done with G4 on the plasmid. Due to the supercoiled nature of the plasmid it is not clear, if G4 structures form and behave the same way on a plasmid as in a single-stranded conformation. Therefore it would be essential to confirm the observed effect with a single-stranded oligonucleotide or linearized plasmids to eliminate the potential that all observed effects are only due to G4 motifs on the plasmid.
Single stranded DNA plasmids are not supercoiled and therefore we do not think the circular nature of our substrates is a problem.

6. Phen-DC has so far only been tested in S. cerevisiae. It is not clear how the ligand acts in a cell free system or in Xenopus. In general it is not clear, if ligands induce or stabilize G4 structures. Therefore, using an alternative ligand such as TMPyP4 would be essential to strengthen their conclusions. In addition, an appropriate and essential control for all presented ligand data would be the use of TMPyP2, which has a similar chemical structure as TMPyP4 but can not induce or stabilize G4 structures.

We have tested the TMPyP4 ligand on G4 and control sequences in a replication assay in extract and found that this compound very inefficiently stabilized G-quadruplex structures (Supplementary Figure S4C and S4D). At higher doses, it inhibits replication likely due to extensive stabilization of non-G4 secondary structures. Importantly, this unspecific replication inhibition is due to the nature of this ligand as the related cationic porphyrin, TMPyP2 induces the same effect (Supplementary Figure S4B). The lack of specificity for the TMPyP4 compound in our system may be due to non-telomeric sequence and context.

7. Figure 3 is hard to understand. The authors argue that the differences they observe might be due to different G4 structures. This needs further explanation and experimental evidence by testing more G4 motifs with similar loop size or similar G-tracts. This observation, also highly relevant, is not discussed in the discussion. It would be very interesting to see if the stability (maybe melting curves can support this observation) or the structure (parallel, antiparallel) of a given G4 is the cause of this difference. An alternative idea would be to test if different monovalent cations (KCl results in strong G4 whereas LiCl in weaker) alter the observed phenomena.

We agree that it would be interesting to gain insight into the role of the G4 type and loop size in replication stalling using our system to match the dozens of biophysical papers that have been published over the last decade. However, doing this properly requires the generation and characterization of a large set of new substrates, and is not adding to the findings and conclusions of this study. To us, it is therefore beyond the scope of this manuscript. We have removed the speculative text in the results section and added a paragraph concerning this issue to the discussion on page 17.

8. On page 12, lane 10 it is not clear what G4G5N is referring too. Therefore, the whole paragraph is not clear and is rather confusing

We have corrected the figure references. We also noticed that Figure 4D was wrongly labeled which might have added to the confusion, and for which we apologize. This is now corrected.

9. Figure 4 nicely illustrates the effect of Phen-DC on G4-dependent replication stalling. It is essential to also show (same conditions as C,D) DNA replication of control samples (non G4) in the presence of Phen-DC.

We have added a sequencing gel using a control sequence (C3P) in the presence and absence of Phen-DC3 to Supplementary Figure 6C.

10. The obtained results are very interesting and clear. To further validate the function of FANCJ and G4 structures and their role during DNA replication it would be essential to check DNA replication at G4 substrates also in the absence of other human helicases such as BLM or WRN. These experiments would strengthen the hypothesis that FANCJ is required for G4 unwinding prior DNA replication and not just simply any given helicase.

We have clearly shown that FANCJ is specifically required to replicate a considerable fraction of G4 substrates since replication of these structures can only be rescued by the addition of FANCJ.
We hope that this reviewer appreciates that the generation and validation of new antibodies to several other helicases (BLM, WRN, RTEL, XPD) requires a tremendous effort and is also uncertain with respect to success. We consider that the generation of these reagents and the functional analysis of other helicases, although very interesting, go beyond the scope of our study.

Minor points:
1. In the abstract the authors state that G4 structures play a role; this is misleading, because to this date it is a hypothesis that G4 structures could be involved in biological processes such as DNA replication, transcription...
We have adjusted the wording in the abstract
2. In the abstract, lane 7, is a spelling mistake: from instead of form.
This is corrected
3. In the whole manuscript the authors switch between G4 and G-quadruplex.
We now consistently refer to ‘G-quadruplex structure’ when referring to the structure and ‘G4 sequence’ when referring to the sequence.
4. It is very confusing for a non G4 person to understand the differences of G4 structures, motifs, sequences, DNA or substrate. A consistent nomenclature, which is introduced and explained, will be beneficial.
We have removed the words ‘motifs’ and ‘substrates’ and have now used only ‘G-quadruplex structure’, ‘G4 sequence’, and ‘G4 plasmid’.
5. In the introduction it will be helpful to rearrange the Figure panels. It will be helpful to cite Figure 1B already in lane 8 when the motif is first introduced.
We have changed this.
6. Only references from human are cited, this should be explicit stated. I don’t understand that.
We have stated more explicit which cell type we refer to.
7. On page 10, second lane of the second paragraph, C. elegans has to be italics.
This has been corrected.
8. The labeling of Figure 4C,D is wrong: both figure panels are labeled with G4 G3N.
This has been corrected.
9. In the result section the author switch tense between past and present. It would be beneficial to stay in present (e.g. page 14)
We have made some adjustments and aimed to used past tense when describing our results and present tense in our conclusions.
10. Total gels of Figure 4 C, D will help to better understand the data in the whole context.
We have added the full gels of Figure 4C and D to Supplementary Figures S6A and S6B.
11. Figure 5D, why is the bottom gel for the Mock sample only 2-40 minutes visualized?
In a mock condition replication stalling is never observed beyond 40 minutes, therefore we did not take time points for this condition. However, we have replaced this experiment with a new experiment in which FANCJ is more extensively depleted (see reviewer # 3), and in which we included these late time points.
12. On figure 5D in both bottom gels a very short band appears in the first lane (2 min)- is this observed in all assays and only visible here due to a different gel processing? What is the nature of this band?
We do sometimes see additional transient and faint bands indicative of replication stalling below the G4-stalled band. These never persist as long as the G4-stalled band and are most likely a result of additional, less stable, secondary structures in the ssDNA plasmids.

Referee #3

In this manuscript, the authors investigate the effects of G quadruplexes (G4) on DNA replication using a nice in-vitro system that allows the replication of single-stranded DNA. They show that DNA synthesis transiently stalls at G4 and suggest that FANC J is involved in the resolution of these structures. This is a technically interesting piece of work, that provides useful information but the
most important conclusions are clearly overstated and could be misleading. More precise comments that could improve this manuscript are below.

1. A major and important criticism concerns the role of FANC J in the resolution of G4 structures during DNA synthesis. The authors state several times that G4 structures are not resolved in the absence of FANC J (see for example page 13, first paragraph). However, all the figures show that up to 90% of these structures are resolved relative to the control. This is emphasized in the comparison of Figure 5B (middle panel) and Figure 5D (lower right panel). Both panels concern the same experiment, one showing that a maximum of 10 % of G4 remained unresolved and the other showing that nearly all G4 were resolved. The statement that FANC J cause persistent stalling at G4 (also written in the summary) is therefore not in agreement with the data shown in the manuscript. In my opinion, this manuscript shows only a mild effect of FANC J depletion and it is difficult to escape from the conclusion that most G4 are also resolved in the absence of FANC J. The title of the manuscript is also misleading.

This reviewer points out that after FANCJ depletion we only observe persistent replication stalling in ~10% of the G4 substrates and that stalling does not persist equally long in all experiments. We would like to clarify two important aspects of our data. First, on a technical note, the effect of FANCJ depletion on the duration of replication stalling depends on the efficiency of depletion. To clarify this we have added an experiment under here showing that more than 2 consecutive rounds of FANCJ depletion are required to see fully persistent replication stalling.

- Figure legend: Xenopus egg extract was mock-depleted, or FANCJ-depleted for 2, 3, 4, or 5 rounds. Depleted extracts were used to replicate the G4-template. Replication products were extracted, separated on 6% urea-PAGE gels and visualized by autoradiography (top). Depleted extracts were analyzed by Western blot using a FANCJ antibody (bottom).

We show in this experiment that the degree of stalling is in agreement with the degree of depletion. After 2 rounds of FANCJ depletions there is some protein left (see Western blot) and the stalled replication product does not persist (autoradiograph). However, after 3 or 4 rounds of depletion the levels of FANCJ are under the detection limit of the antibody and the stalled band stably persists until the latest time point.

In retrospect, we may not have chosen the best experiment for Figure 5D, it was one of our earlier experiments in which we used only 2 rounds of depletion. While it served our aim to show the effect...
of Phen-DC3 on replication stalling, it was not the best FANCJ depletion. We have repeated this experiment using more extensive FANCJ depletion conditions to show that replication stalling is persistent in the absence of FANCJ (see new Figure 5D).

This brings us to the raised concern that not all G4 structures in a given population seem to be substrates for FANCJ. We agree that there are likely other mechanisms to resolve G4 structures during DNA replication, in addition to the FANCJ-dependent mechanism we here describe. We clearly addressed these in the discussion, and in Figure 7 and had no intention to be misleading. We now adjusted some of the phrasing to be more clear on this issue. Our genome contains more than 300,000 potential G4 sites, even if only a small fraction of those form a G-quadruplex at a given time, there would still be a considerable number of G4 structures causing problems in the absence of FANCJ. Therefore we consider this FANCJ-dependent mechanism highly relevant.

2. Based on Figure 3 data, it is difficult to state in the text and figure legend that the stalled band is at 760 nt from the G4 as the shown gels did not allow a good resolution. Moreover, no molecular weight makers are shown. Figure 4 gives the correct information, but the presentation of the results should be reformulated to avoid premature conclusions that are not in agreement with the shown data.

We have added a gel with size markers to Supplementary Figure 3 and adjusted the text accordingly.

Other comments:

. Figure 4B should indicate in the ordinate the size of some of the corresponding bands in the sequencing ladder.
This has now been included in Figure 4B.
. Page 12: and corresponding Figure 4D. I guess that the gel corresponds to G4G23, and not G4G3N. In addition, the text refers to G4G5N. This part should be checked.
We have corrected the labeling of the Figure 4D and adjusted the text.
. Figure 7 does not bring any new information.
We favor the inclusion of figure 7: in addition to summarizing our study in a visual way, we think it is relevant because it also addresses the exact point this reviewer is making above, namely that there are other mechanisms to resolve G4 structures.

Editorial Decision 06 August 2014

Thank you for submitting your revised manuscript for our consideration, and apologies for the seasonal delay in its reevaluation. It has now been seen once more by all three original referees (see comments below), and I am happy to inform you that they consider the revised manuscript satisfactorily improved and retain no further objections towards publication in The EMBO Journal.

Before formal acceptance of the paper, I would like to ask you to consider the remaining suggestions of referee 3 and incorporate them into the manuscript text where applicable (further figure changes would not be needed in my opinion). You could then simply send us a modified text document file via email. Regarding the well-taken title change proposed by referee 3, my suggestion would be "FANCJ promotes DNA synthesis through G quadruplex structures".

After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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Referee #1

The authors have addressed my points adequately.
Referee #2

The Authors have addressed all my major concerns, therefore I highly recommend this article for publication.

Referee #3

I found this revised manuscript well improved and deserving publication. If accepted, I recommend the authors to take care of the following recommendations in the final version of the manuscript.

The authors should precise in the summary and also in the introduction that they used single stranded DNA templates. It is not clear for everybody, including one of the reviewers, that the plasmids used were single strand DNA plasmids.

Conclusion of paragraph 1, "that G-quadruplex structures in ssDNA plasmids cannot be bypassed by a polymerase alone" is overstated as the data are obtained only with a bacteriophage DNA polymerase. Unless demonstrated by the authors or other authors for eukaryotic DNA polymerases, this sentence should be modified.

From the experiment shown in Figure 5C, it is not possible to conclude that the replication machinery remained at the fork in absence of FANCJ. It might have dissociated but could reassociate again, as the template is still a ss DNA containing a primer. The corresponding statement in the discussion should be also modified.

Figures 1 and 2 can be grouped.

In my opinion, a more suitable title should be: "FANCJ helps DNA synthesis through G quadruplex structures". I believe it is more correct to claim that DNA synthesis was analysed, as opposed to DNA replication, which is more related to ds DNA template