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G4 motifs affect origin positioning and efficiency in two vertebrate replicators

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 December 2013

Thank you for submitting your manuscript on G4 regulation of vertebrate replicators for our consideration. We have now received the below comments of three expert referees, and I am pleased to inform you that all of them find your results of interest and potential importance. Pending adequate addressing of a limited number of specific concerns, we shall therefore be happy to consider a revised version of your manuscript further for publication in The EBMO Journal.

As you will see, the main concerns of the reviewers lie with the decisiveness of the evidence for these G4 structures also existing in cells (ref 2), and directly affecting replication initiation instead of leading to SNS enrichment due to fork pausing (ref 3). Although I realize that these issues may be difficult to tackle experimentally, I would appreciate hearing back from you within the next few days how you could envision responding to these concerns, in order to be able to discuss the requirements for a successful revision of this work.

I should add that it is our policy to allow only a single round of major revision and that it is therefore important to carefully answer to all points at the stage of resubmission. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community. 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.

Thank you for the opportunity to consider this work for The EMBO Journal - I look forward to hearing from you and to receiving your revision!

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REFEREE REPORTS:

Referee #1:

The search for sequence based replicators has been a holy grail for the mammalian replication field. In vitro, any sequence can be replicated in Xenopus extracts, and plasmid based assays have also exhibited very promiscuous replication (just about any sequence tested is capable of replicating). However, in the last few years the advent of genome-wide approaches have detected enrichment of small nascent strands (SNS - a replication intermediate) in the vicinity of G-quadruplexes. Given their marked specificity for accumulation of nascent strands it is actually quite surprising that they were missed before. A caveat though is that they are only detected by one method - lambda exonuclease digestion of nascent strands. Which beg's the question - are they real or an artifact of the assay?

The critical advance in this manuscript is that the authors attempt to address this by using the dt40 chicken cell recombination system where they can delete and re-orient the G-quadruplex structures. Clearly, the accumulation of SNS intermediates is G4 dependent as the authors have very nicely shown. However, they could still be an artificat of the approach used (perhaps resistance to lambda exo) or another structure (stalled forks). To address this concern, the author's go further and use a second cruder assay (changes in replication timing) to assess the in vivo function of these structures. Although the impact on timing were much more subtle, they do support the authors claims that G4 structures participate in DNA replication. This work represents a critical advance and is the most rigorous test to date of G4 structures.

Note: I have reviewed this manuscript elsewhere and my prior experimental criticisms have already been addressed in this revision.

Referee #2:

A.L. Valton et al.., G4 motifs affect origin positioning and efficiency in two vertebrate replicators

The “holy replicator" probably belongs to the least understood functional elements of the eukaryotic chromosome. Unlike in yeast, no consensus sequences but rather a number of structural features are found in the replication origins of higher eucaryotes. Moreover, it is believed that many more genomic sequences have the potential of being an origin than are actually used in the cell. Therefore our current idea is that selection and activation of an origin of replication depends on poorly understood epigenetic features, such as chromatin structure, interplay between transcription and replication up to the localization in the cell nucleus. Last year, a genome wide study revealed that the majority of Drosphila and mammalian origins contain sequence elements that have the potential to form G-quadruplex structures. In last 10 years evidence accumulated that these structures can form in vivo and they are believed to have regulatory functions in a variety of biological processes, such as regulation of transcription, telomere structure, translational control and others. Deletion of helicases able to unwind G4 lead to a deletion of genomic regions that contain G4, suggesting that this structure can form during replication and that helicases are required to resolve them in the course of replication. The observation of G4 in origins now led to the hypothesis that this unusual structure may also be involved in regulating origin activity. This hypothesis was supported by the fact that the human origin recognition complex preferentially binds to G4 motifs in single stranded DNA. Testing the hypothesis that G4 structures are actually involved in the initiation of replication is of great biological relevance.

Valton et al. test this hypothesis on two well defined origins of replication, the chicken bA and a chicken origin located upstream the gene encoding med14. Both origins contain several motifs able to form G-quadruplex structures. By deleting or mutating these motifs they clearly show in a series of well designed and performed experiments that the integrity of these motifs is required for replication initiation. In one origins two G4 motifs cooperate while the other contains only one critical G4 motif but needs in addition the cooperation of another cis-regulatory element. They also show that the mutations made do not affect the binding of a transcription factor but rather stability of a potential G4 structure, but of course they can not exclude binding of other trans-factors to these

motifs. Therefore, this paper describes nicely a correlation between sequence motifs that can form G4 structure and origin activity.

The major limitation of this paper is, similar to most other papers on G4 structures, that they do not show that the G4 motifs actually form G4 structures in the cell. Having now so many ligands specifically binding to G4 structures as well as specific antibodies directed against it in our hands, I wonder whether this question could not be addressed experimentally. Another possibility could be to use ligands specifically stabilising G4 structures in the cell and ask for the effect on replication control. Having any experimental evidence for the existence of this structures in these origins in vivo would make this paper to an outstanding work. As it is it is a correlation. The authors are fair enough to write that G4 motifs (not G4 structures) affect origin positioning and efficiency but in all figures it is implied that this structure actually exists.

If formation of G4 structure is required for regulation of replication initiation it has to be tightly regulated. So far, we have no idea how this could be achieved and this might be discussed.

To me it is not obvious from this work how G4 structure could be involved replication regulation and any mechanistic model or hypothesis would be most desirable.

Minor concerns

1. p. 6; Fig.2: Why is SNS enrichment in the modified allele much higher than in the control?

2. The paper is sometimes difficult to read, mainly because the text does not always refers to the Figure, does not explain why a conclusion is drawn and the figure legends are very puristic and could go into more detail.

In summary

This is an experimentally sound and informative work showing clearly a correlation between G4 structure stability in vitro and origin activity. It thus describes a novel potential biological function of this unusual DNA structure. However, it fails to demonstrate the existence of this structure in vivo, either directly or by disturbing this structure by G4 binding ligands and testing their effect or any other indirect evidence for the existence of this structure. Finally, a number of issues (regulation of G4 structure formation, possible effect of this structure on origin activity) should be addressed.

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This manuscript deals with currently rather controversial issue on the nature of vertebrate replication origins. The authors utilizes chicken DT40 cells to manipulate the G4 motifs found in the vicinity of the putative replication origins and evaluate their potential roles in initiation of DNA replication. Authors use short nascent strands enrichment technique throughout this study as a method to identify the initiation site.

Disruption of G4 structures resulted in the decreased enrichment of SNS. The effect of G4 on enrichment of SNS is dependent on its orientation. On the basis of these results authors conclude that G4 (plus some additional element) plays crucial roles in replication initiation.

The experiments are generally conducted in a proper manner within the limit of the method and the interpretation appears to be consistent with the conclusion.

However, I feel that all the results are perfectly consistent with another explanation that G4 is pausing the leading strand synthesis, resulting in the enrichment of the SNS only on one side of the G4. It is well accepted that origin firing in eukaryotic genome is highly stochastic and probably virtually any sequence can function as replication origins, given such an opportunity, although probability of firing could be affected by the nearby chromatin structures, transcription and other factors. It is conceivable that G4 can cause temporal fork pausing even in the presence of Pif1 and other G4-unwinding enzymes. Authors notice this possibility and state that it is unlikely that fork pausing at G4 causes the enrichment of SNS. In spite of these argument, I find it difficult to exclude

it completely, since currently there is no biochemical evidence that links G1 structures to pre-RC formation or origin activation.

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In order to provide more persuading evidence for the authors' conclusions, it would be necessary to conduct independent assays of replication initiation on the mutant origins,e.g. DNA fiber analyses combined with FISH, although I acknowledge that those are not very easy experiments to conduct. Alternatively, I want to see some biochemical evidence that G4 facilitates a process of replication initiation (e.g. pre-RC formation or activation step of pre-RC).

1st Revision - authors' response 07 January 2014

We thank you for your agreement in receiving a revised manuscript of our paper entitled “G4 motifs affect origin positioning and efficiency in two vertebrate replicators” and for your careful reading of our arguments regarding the possibility to correctly answer to referees’ concerns.
We have made a point-by-point response to referees’ comments. We have incorporated our new unpublished results on origin interference as additional supplementary figures. We also explain more clearly why our timing assay was consistent with the identification of replication origin but not with the mapping of replication pausing sites.

Referee #1:

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as regulation of transcription, telomere structure, translational control and others. Deletion of helicases able to unwind G4 lead to a deletion of genomic regions that contain G4, suggesting that this structure can form during replication and that helicases are required to resolve them in the course of replication. The observation of G4 in origins now led to the hypothesis that this unusual structure may also be involved in regulating origin activity. This hypothesis was supported by the fact that the human origin recognition complex preferentially binds to G4 motifs in single stranded DNA. Testing the hypothesis that G4 structures are actually involved in the initiation of replication is of great biological relevance.

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The major limitation of this paper is, similar to most other papers on G4 structures, that they do not show that the G4 motifs actually form G4 structures in the cell. Having now so many ligands specifically binding to G4 structures as well as specific antibodies directed against it in our hands, I wonder whether this question could not be addressed experimentally. Another possibility could be to use ligands specifically stabilising G4 structures in the cell and ask for the effect on replication control. Having any experimental evidence for the existence of this structures in these origins in vivo would make this paper to an outstanding work. As it is it is a correlation. The authors are fair enough to write that G4 motifs (not G4 structures) affect origin positioning and efficiency but in all figures it is implied that this structure actually exists.

We understand the concerns of reviewer 2 related to a lack of direct evidence of G4 formation at the G4 motifs we have studied. He/She suggested using either G4 antibodies or specific G4 ligands to further improve our work so that it would become "*an outstanding one*". We would like to discuss the feasibility of these experiments, their expected results and potential conclusions, as the referee itself "*wonders whether this question could not be addressed experimentally*".

G4 antibodies: several reports published in 2013 used G4 antibodies to explore the formation of DNA or RNA G4 in human cells (Biffi *et al* Nat Chem 2013a &b, Henderson *et al* Nucleic Acids Res 2013). These studies were based on immunofluorescence and reported the formation of G4 foci in nuclei or cytoplasm under different experimental conditions (use of G4 ligands, helicase mutants). Due to the limited number of foci, these antibodies (1H6 and BG4) detect at best either some specific sites or clusters of G4 motifs, after permeabilization and fixation of cells. In our opinion, these experiments only provide an additional indirect evidence of G4 formation to those provided earlier by the group of J-F Riou using specific G4 ligands that promote G4 stabilization (telomestatin or tritiated 360A) (Gomez *et al* JBC 2004 & Granotier *et al* Nucleic Acids Res 2005) since it is still unclear whether these antibodies bind native G4 structures or also promote their stabilization. In addition, these experiments did not determine the location of G4 motifs, excepted for telomeric sequences. Another publication (Lam *et al* Nature Commun 2013) used another antibody (hf2) to pull-down G4 motifs *in vitro* from purified genomic DNA. We think that this

publication lacked the critical controls (at least exonuclease treatment to digest telomeric G-overhang and other controls to prove that G4 structure really occurred in a duplex DNA context). It is conceivable to use one of these antibodies to perform pull-down or ChIP experiments (except 1H6 for which it is indicated that attempts to perform ChIP experiments failed). However, the limited number of G4 motifs pulled-down from the *in vitro* experiment raised the question of the antibody selectivity towards the G4 motifs we used in our study. The *in vitro* Kd or competitions experiments also indicated that these antibodies did not recognize all possible G4 structures. If we can obtain one of these antibodies (which is not sure), we will have to check its Kd again on our sequences. In addition, since it is generally accepted that the formation of G4 structures *in vivo* is a transient process temporally coupled to functional processes (DNA replication or transcription). It therefore might be difficult to catch these structures. We will have to find the conditions to synchronize cells at the onset of S phase or in G1 (phase of origin licensing), find a way to introduce the antibody without disturbing the cellular metabolism and the nucleoprotein structure, perform the cross-link or the pull-down with all the required controls to be sure that we selectively get the firing origins. Clearly, such work is not in the scope of our study and will require considerable efforts with uncertain results. The referee mentioned that "*similar to most other papers on G4 structures, they do not show that the G4 motifs actually form G4 structures in the cell*". The main reason is that so far there is no tool available to address correctly this question.

G4 ligands: the use of specific G4 ligands is potentially more interesting. We have in our hands one of the most specific derivative designed to date, 360A, with obvious evidence of its action at telomeres, during transcription, splicing and translation of mRNA (Halder *et al* BMC Res Note 2012, Gomez *et al* Nucleic Acids Res 2004, Gomez *et al* Nucleic Acids Res 2010, just to cite the publications of J-F Riou's group). However, we wonder about the expected results on origin activity in DT40 cells treated with G4 ligands since their effects might be pleiotropic and/or indirect. As an example, the group of J-F Riou already published a study on the transcriptional effect of 360A, showing that treatment of HeLa cells lead to transcriptional deregulation with up-regulated or down-regulated genes being enriched in G4 motifs (Halder et al 2012). The same group had further pursued this study to analyze the transcriptional effect of 360A (with shorter period of treatment) in a cellular model sensitive and its counterpart resistant to this compound (to be presented at the next telomere meeting in April 2014, C. Trentesaux, S. Chowdhury and J.F. Riou). Again, the group observed a highly significant enrichment of G4 motifs within genes transcriptionally modulated upon treatment with 360A (identical results were found with 4 other G4 ligands). Resistance to 360A lowered the absolute number of genes modulated by the ligand and a transcriptional adaptation was found in resistant cells affecting loci enriched in G4 motifs (81% of differentially expressed genes with $p<E-300$). However, we are still unable to understand why transcriptional modulation triggered by 360A at a specific locus is either up- or down-regulated. Finally, the common genes modulated by the 5 ligands are very limited, suggesting either an *in cellulo* uncoupling for G4 motif selectivity (which is surprising since G4 motif selectivity is very limited *in vitro*) or a cascade of indirect effects on DNA/RNA metabolism due to the duration of the treatment. If we test the impact of 360A (the best characterized ligand in our hands) on replication origin firing, we will probably encounter similar problems. Since 360A may modulate simultaneously DNA replication (through both initiation and fork progression), transcription, splicing, translation and the binding of specific proteins to single-stranded or G4 DNA, we think that conclusions drawn from such experiments are limited. We already know that another group working on the same hypothesis (group of Marcel Méchali) is currently studying the effect of G4 ligands on replication origin firing. Their data presented this year at several meetings indicated that as observed for transcription some origins are up- or down-regulated, while others are unchanged. So, to conclude on this point, it is of course possible to treat DT40 cells with 360A and to analyze the modulation of origin firing. However, we don't know what will be the final results and the conclusions that can be drawn. During the time required for the review process, we think that we will only be able to provide preliminary conclusions, as compared to the present work that was initiated more than 4 years ago and provide clear and new important insights.

Biffi G, Tannahill D, McCafferty J, Balasubramanian S. Quantitative visualization of DNA G-quadruplex structures in human cells. Nat Chem 2013;5:182-6.
Lam EY, Beraldi D, Tannahill D, Balasubramanian S. G-quadruplex structures are stable and detectable in human genomic DNA. Nat Commun 2013;4:1796.

Biffi G, Di Antonio M, Tannahill D, Balasubramanian S. Visualization and selective chemical targetinh of RNA G-quadruplex structures in the cytoplasm of human cells. Nat Chem 2013;DOI: 10.1038/NCHEM.1805
Henderson A, Wu Y, Huang YC, Chavez EA, Platt J, Johnson FB, et al. Detection of G-quadruplex DNA in mammalian cells. Nucleic Acids Res 2013.
Halder, R., Riou, J.F., Teulade-Fichou, M.P., Frickey, T. and Hartig, J.S. (2011) Bisquinolinium compounds induces Quadruplex-specific transcriptome changes in HeLa S3 cell line. *BMC Res Note*, **5**, 138.
Gomez, D., Guédin, A., Mergny, J.L., Salles, B., Riou, J.F., Teulade-Fichou, M.P. and Calsou, P. (2010) A G-quadruplex structure within the 5'UTR of the TRF2 mRNA represses translation in human cells. *Nucleic Acids Res*, **38**, 7187-98.
Granotier, C., Pennarun, G., Riou, L., Hoffschir, F., Gauthier, L.R., De Cian, A., Gomez, D., Mandine, E., Riou, J.F., Mergny, J.L., Mailliet, P., Dutrillaux, B. and Boussin F.D. (2005) Preferential binding of a G-quadruplex ligand to human chromosome ends. *Nucleic Acids Res*, **33**, 4182-4190.

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To me it is not obvious from this work how G4 structure could be involved replication regulation and any mechanistic model or hypothesis would be most desirable.

We have described in more details hypotheses on the regulation of G4 formation and on how G4 structures may be involved in origin function in the discussion (page 16, first paragraph).

Minor concerns

1. p. 6; Fig.2: Why is SNS enrichment in the modified allele much higher than in the control?
To rigorously compare our mutants, we firstly created a WT version of the med14 origin containing the 193bp fragment that remained after excision by the Cre recombinase of the gene of selection (Blasticidin). This fragment allowed us to differentiate the modified allele from the WT allele. This fragment is located only 300bp away from G4#5 and therefore may have an impact on origin function (Figure 2A). We have shown on Ori b^A that deleting flanking regions had an impact on origin function (either stimulatory or inhibitory, Figure 3). On Ori med14, we found on two independent clones that, as mentioned by the referee, SNS enrichment was higher on the modified allele (Figure 2B). Then mutants deleted for G4 #4 and G4 #5 or both were created. These mutants also contained the 193bp fragment and therefore can be compared with the "WT modified version".

2. The paper is sometimes difficult to read, mainly because the text does not always refers to the Figure, does not explain why a conclusion is drawn and the figure legends are very puristic and could go into more detail.
We have added additional information in the legends of several Figures (1, 2, 7 and 8) and have tried to refer to the figures as much as possible to facilitate the reading of the paper.

In summary

This is an experimentally sound and informative work showing clearly a correlation between G4 structure stability in vitro and origin activity. It thus describes a novel potential biological function of this unusual DNA structure. However, it fails to demonstrate the existence of this structure in vivo, either directly or by disturbing this structure by G4 binding ligands and testing their effect or any other indirect evidence for the existence of this structure. Finally, a number of issues (regulation of G4 structure formation, possible effect of this structure on origin activity) should be addressed.

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vicinity of the putative replication origins and evaluate their potential roles in initiation of DNA replication. Authors use short nascent strands enrichment technique throughout this study as a method to identify the initiation site.

Disruption of G4 structures resulted in the decreased enrichment of SNS. The effect of G4 on enrichment of SNS is dependent on its orientation. On the basis of these results authors conclude that G4 (plus some additional element) plays crucial roles in replication initiation.

The experiments are generally conducted in a proper manner within the limit of the method and the interpretation appears to be consistent with the conclusion.

However, I feel that all the results are perfectly consistent with another explanation that G4 is pausing the leading strand synthesis, resulting in the enrichment of the SNS only on one side of the G4. It is well accepted that origin firing in eukaryotic genome is highly stochastic and probably virtually any sequence can function as replication origins, given such an opportunity, although probability of firing could be affected by the nearby chromatin structures, transcription and other factors.
It is conceivable that G4 can cause temporal fork pausing even in the presence of Pif1 and other G4-unwinding enzymes. Authors notice this possibility and state that it is unlikely that fork pausing at G4 causes the enrichment of SNS. In spite of these argument, I find it difficult to exclude it completely, since currently there is no biochemical evidence that links G1 structures to pre-RC formation or origin activation.

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Although authors present another set of assay of measuring the replication timing of the ectopic segment surrounded by the USF binding sites, I found the results not compelling enough to support authors' claim that G4 facilitates the initiation.

As mentioned by the referee # 3, we were highly concerned by the possibility that the SNS assay might catch preferentially pausing sites found nearby origins. If it were the case then those origins would be abnormally enriched in the SNS fraction. We have clear indications that it is not the case.
Firstly, functional studies on the b^A origin showed that deletions of regions flanking the G4 motif (but not inside the motif) modulated SNS enrichment with one deletion reducing the enrichment to background level (200 bp deletion 3' of the G4 motif). The hypothesis of a pausing site would imply that in this mutant the flanking region has an impact on the G4 stability which we agree remains an option.
To go further into our demonstration, we have designed a new origin assay that is based on the observation made in a previous study (Hassan Zadeh et al, 2012). We have found that a strong origin flanked by USF binding sites can shift to earlier replication a locus replicated in mid-late S-phase. The assay gives a significant timing shift corresponding to about 45 min which can be considered as subtle (as mentioned by referee #1) but is highly robust since we systematically obtain similar shifts on two independent clones made for each construct. **Moreover this assay cannot detect a sequence that would impede replication fork progression since a pausing would shift the replication timing toward later replication.** We previously showed that this replication timing shift depend both on having an origin and on flanking the origin with USF binding sites. We therefore used this assay to compare origins (Wt or mutated) giving a strong SNS enrichment with origins (mutated) deeply affected in their SNS enrichment. We clearly showed that on 6 independent clones displaying a high SNS enrichment, the replication shift is detectable whereas on 8 independent clones having a low SNS enrichment the replication timing shift is not detectable (Figure 8 of our manuscript). It should be noted that in contrast to referee #3, referees #1 and 2 are convinced by our timing assay.
To convince referee #3, we added previously published and new results that altogether reinforce our demonstration (New Supplementary Figures 11, 12 and 13). We constructed a cell line containing on both alleles the minimal transgene that gives a significant shift (ori b^A flanked by USF binding sites) linked to a strong promoter (b-actin) that drives expression of a gene of selection (Blasticidin on one allele and Puromycin on the other one) (Supplementary Figure S11A). This larger construct gives a strong shift from mid-late to mid-early S phase (Hassan Zadeh et al, 2012). We previously asked the question whether the early replication timing shift we observed at the site of integration

extended into flanking chromosomal regions. We showed in this cell line that the global direction of replication forks went from the transgene toward flanking regions since the timing of replication became progressively later upstream and downstream of the site of insertion (Supplementary Figure S11B). This contrasted with a wild type strain in which the replication timing was almost similar along a 300 kb region centered on the site of insertion (Supplementary Figure S12B). This result suggested that the b^A origin inside the transgene is active in most cells and that timing control elements surrounding this origin imposed locally the timing on this origin. The closest sites of SNS enrichment, detected by deep sequencing and validated by qPCR, are located 58 kb upstream and 80 kb downstream of the site of insertion (Ori L and Ori R, Supplementary Figure S12A and S13B (WT)). We have tested the impact of the double insertion of this strongly shifted transgene on SNS enrichments of flanking sites. We found that SNS enrichments dropped from 250% and 197% in the WT cell line to 71% and 68% in the modified cell line at OriL and OriR respectively (Supplementary Figure S13B). These two sites also contained G4 motifs on both strands (Supplementary Figure S13A). If we follow the hypothesis of referee #3, we would have to admit that the introduction of our transgene (containing a hypothetic pausing site) between two additional hypothetic pausing sites lower fork pausing at these sites for an unknown reason. This hypothesis is not consistent with our new observation. In contrast, our results fitted perfectly well with the reduction of origin firing at Ori L and Ori R by a mechanism of origin interference. OriL and OriR that normally fired later than the new inserted origin were passively repressed by replication forks emerging from this new replicator (Supplementary Figure S13A).

In order to provide more persuading evidence for the authors' conclusions, it would be necessary to conduct independent assays of replication initiation on the mutant origins,e.g. DNA fiber analyses combined with FISH, although I acknowledge that those are not very easy experiments to conduct. Alternatively, I want to see some biochemical evidence that G4 facilitates a process of replication initiation (e.g. pre-RC formation or activation step of pre-RC).

Since referee#3 is not convinced by our new origin test, he proposed us to use DNA molecular combing combined with FISH to study the dynamic of origin at our modified locus. Only a few laboratories in the world have performed these experiments on single loci and we know that even for specialists this type of investigation is extremely time consuming and therefore would prevent us from giving an answer in the three months delay provided for a revision.
Referee #3 also asked for biochemical evidence that G4 facilitates a process of replication initiation. As mentioned in our manuscript and nicely emphasized by referee #2, a recent paper (Hoshina et al, 2013) provides biochemical evidence that G4 structures on RNA or SS DNA are preferentially bound by ORC in vitro.

Hassan-Zadeh V, Chilaka S, Cadoret J-C, Ma MK-W, Boggetto N, West AG & Prioleau M-N (2012) USF binding sequences from the HS4 insulator element impose early replication timing on a vertebrate replicator. *PLoS Biol.* **10:** e1001277

Hoshina S, Yura K, Teranishi H, Kiyasu N, Tominaga A, Kadoma H, Nakatsuka A, Kunichika T, Obuse C & Waga S (2013) Human Origin Recognition Complex Binds Preferentially to G-quadruplex-preferable RNA and Single-stranded DNA. *J. Biol. Chem.* **288:** 30161–30171

