Dear Dr. Royer,

My co-author, Dr. Griffith, and I hereby resubmit for publication a revised version of Manuscript ID bi-2014-006249 “TRF1 and TRF2 Differentially Modulated Telomeric and Non-Telomeric D-loop Formation In Vitro”.

Dr. Griffith and I greatly appreciate your efficient handling of our paper and are in debt to the two reviewers who gave their time to provide suggestions which will significantly enhance the paper and presentation of the results. We note that the first reviewer felt that the work was highly appropriate for Biochemistry with only minor alterations. The second reviewer was also very supportive other than one point which we will comment on below. We have revised the paper to address each of their points and have also made the corrections requested by the editorial office. We are very hopeful that you will find these satisfactory and that the work will now be suitable for publication in Biochemistry.

The most substantial modification to the manuscript includes the addition of sections to the discussion to address the concerns of Reviewer 2. We have also modified the results section to draw the reader’s attention to important findings and modified several figures or figure legends for clarity including substantial revisions to Supporting Information Figure 3 to address a concern from Reviewer 1. You may find a copy of the manuscript with all added or significantly modified sections marked in red titled ‘Manuscript\_Revisions’ submitted as ‘Other Files for Editor Only’.

We have taken the below step to address Reviewer 1’s comments and concerns:

1. “p 9 lane 15: is it a Biorad apparatus?”

*We have addressed this concern by correcting the Imaging paragraph of the experimental procedures section to explain that the gels were imaged using a General Electric Typhoon 9400 scanner.*

1. “Figure S2 legend: In the title, the authors mention that TRF2 required telomeric sequences to mediate D-loop formation. Since the levels of D-loop formation obtained for TRF2 using telomeric sequences are already low, would it be possible to detect a lower activity for non-telomeric sequences? The same applies for the main text p10 lane 35.”

*The sensitivity of the displacement-loop (D-loop) assay described in this paper is limited by the level of spontaneous complex formation between substrates and templates (i.e. in the absence of TRF2 or Rad51), which was approximately 0.5%. Variability in the D-loop assay complicates attempts to detect significant differences in complex formation below approximately 1% with the low N’s imposed by the complicated, time-consuming and time sensitive nature of the assays. In Supporting Information Figure 2 several of the substrate/template combinations did not yield reliably quantifiable complex formation. We have added an explanation for why we omitted these results to the figure legend.*

1. “Many labels in Figures are illegible because too small, especially in Supplementary figures, where, to my knowledge, there should be no space constrains...”

*We addressed this concern by increasing the font size in the supporting information figures.*

1. “Supp Fig S3B. It’s not clear to me what are the “3” and the “4” labels above the gel lanes. What’s the “T0+3h” samples? From the diagram S3A, it seems that the samples are all assessed at T0+4hrs. If this is not the case, the authors should clarify.”

*We have addressed this concern by revising the reaction diagram in Supporting Information Figure 3 and the gel and chart labeling to clarify that portions of the reactions are collected at both the 3-hr and 4-hr time points. We have also edited the figure legend to better explain the data.*

We have taken the below steps to address Reviewer 2’s comments:

1. “The first observation strongly contradicts established observations that TRF2 promotes t loop formation and specifically addition of recombinant TRF2 to nuclear extracts appears to restore telomeric D loop formation. This raises questions if what they observe in vitro (that TRF2 inhibits Rad51 mediated telomeric D loop) is relevant. Given that authors recognize this and suggest that other in vivo factors may play a role and in addition, the in vitro assay could be limited ("templates and substrates used are different from their in vivo analogs"), the conclusion that TRF2 inhibits Rad51 mediated telomeric D loop is too premature. More mechanistic work needs to be done to support that conclusion.”

*We addressed this concern by adding sections to the discussion that explain the technical advantaged and limitations of our in vitro assays compared to the nuclear extract work referenced by Reviewer 2, and mention several methodological differences between these assays that complicate direct comparison of their results. In this section we also mention that those nuclear extract results appear at odds with a recent report from the laboratory of Dr. Eric Gilson, which shows that expression of TRF2 hinders HR-mediated processes in vivo. We have also added a section to the discussion wherein we compare out results with other TRF2 characterizations and propose a mechanism that may underlie our observation that TRF2 inhibits Rad51-mediated telomeric D-loop formation.*

1. “Author show that TRF2deltaM does not inhibit Rad51 telomeric D-loop formation. Is it due to the loss of dsDNA binding ability? Author show in a table that the mutant has decreased telomeric DNA binding”

*We report that TRF2ΔM actually promotes Rad51-mediated non-telomeric D-loop formation. This suggests that TRF2ΔM retains some activities vis-à-vis D-loop formation regardless of it’s impaired dsDNA binding. We have addressed this concern by adding a sentence to the results section to bring the reader’s attention to this interesting finding. We further address this concern by adding further discussion of the TRF2ΔM results to the discussion section.*

1. “Is the substrate used for the binding assay the same as what is used in the telomeric D loop formation assay, which is the supercoiled plasmid template?”

*The template used in the EMSA and binding competition assays was a Cy3-labeled PCR product amplified from the pBB that contained the 103 bp telomere tract of pBB, which was the homologous target of the T90 substrate in the D-loop assays. This was explained in detail in the experimental procedures section, briefly mentioned again in the results section, and illustrated graphically and verbally in Figure 2E. We have addressed this concern by adding explicit explanations of the nature of the template in the results section and in the legend of Figure 2.*

1. “While the authors make interesting conclusions such as TRF2deltaM promotes nontelomeric D loop formation but has no effect on telomeric D loop formation, no further explanation or speculation as to what mechanisms underlie these differences.”

*We addressed this concern by editing the results and discussion sections to draw the reader’s attention to the observation that although TRF2∆M did not promote Rad51-mediated telomeric D-loop formation across the entire Rad51 concentration range tested, TRF2ΔM did significantly promote Rad51-mediated telomeric D-loop formation at the lowest Rad51 concentration tested (Figure 3B). We speculate that the high efficiency of telomeric D-loop formation in reactions with high Rad51 concentrations may mask the ability of TRF2ΔM to promote this activity. Additionally, we have added a section to the discussion that both examines possible mechanisms by which TRF2ΔM may promote D-loop formation in vitro and which points out how these processes may explain how TRF2 may promote HR in vivo.*

1. “Finally authors also suggest that TRF1 promotes Rad51 mediated telomeric D loop formation. Author suggest that the "ability of TRF2 to inhibit this process is not simply due to Myb domain binding" but again fail to speculate what could be the mechanism for TRF2 inhibition of Rad51 mediated telomeric D loop formation.”

*We sought to address this concern in parallel with Reviewer 2’s concerns about our TRF2 results. We have added a section to the discussion wherein we observed that TRF1 does not promote supercoiling within telomeric dsDNA and does not inhibit Rad51-mediated telomeric D-loop formation, in keeping with our model that the inhibition is mediated by supercoiling. In this section we also observe that TRF1’s oligomeric binding is fundamentally different from TRF2, and TRF1’s ability to promote Rad51-mediated telomeric D-loop formation may stem from its lower-order binding characteristics and its inability to promote supercoiling.*

We have taken the below steps to address the concerns of the editorial office:

1. “Relocate Supporting Information paragraph before the References.”

*We have complied with this instruction and have moved the Supporting Information paragraph before the references.*

1. “Provide a graphic for use in the Table of Contents.”

*We have complied with this instruction and have added a 1.375” x 3.5” 300 dpi color TIF illustrating a reaction scheme wherein TRF2 inhibits Rad51-mediated telomeric D-loop formation to the final page of the manuscript.*

Regards,

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