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A DNA polymerization-independent role for mitochondrial DNA polymerase I-like protein C in African trypanosomes

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MS TITLE: Structure-function analysis reveals a DNA polymerization-independent role for mitochondrial DNA polymerase IC in African trypanosomes.

AUTHORS: Jonathan C. Miller, Stephanie B. Delzell, Jeniffer Concepcion-Acevedo, Michael J. Boucher, and Michele M. Klingbeil
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have prepared a manuscript that describes analysis of a trypanosome kinetoplast polymerase - POLIC. They use an interesting simultaneous RNAi and expression of dead or truncated versions of POLIC system to study the functions POLIC and its domains. POLIC has a conserved family A DNA polymerase domain (POLA) and the uncharacterized N-terminal region (UCR). They show that RNAi complementation with wild-type POLIC restored kDNA content and cell cycle localization but, point mutations in the POLA domain impaired minicircle replication. Complementation with the POLA domain alone abolished POLIC foci formation and partially rescued the RNAi phenotype. And, they suggest that the UCR is implicated localization and segregation of kDNA daughter networks.

The work is well thought out and provides new insights. The experiments are clear, relevant and precise and the results are novel and important to the scientific community. The level and quality written English is of a high standard and the references are appropriate, although the figure legends need attention (see below). The article is also useful to non-specialist, not too long and is appropriate. The work is of high enough quality and novelty to be published in J. Cell. Science, but only if the points below are addressed correctly.

Comments for the author

Critiques;

Major

1. Line 209 211 and other places, the authors state that; “Taken together, IC-UTR silencing phenocopied the previously reported LOF, and kDNA replication defect but also displayed clear signatures of a segregation defect”. Implying that knockdown of POLC disturbs kinetoplast segregation. This is incorrect because the authors did not interfere with the kinetoplast segregation machinery per se, which, as they mention, is a basal body based segregation machinery. Unless, they have basal body/flagella markers or measurements to prove this. From the data they have provided in the manuscript, in my humble opinion, they have interfered with the structure or component of the network that is attached to the segregation machinery. Thus, ancillary or components of kinetoplasts as well as marker proteins such as TAC 102 are floating around in the mitochondrion matrix. Can they please rethink the text and find a replacement for their idea of segregation with something else? Alternatively provide evidence that the segregation machinery is affected by POLIC knockdown.

2. Results in lines 319-320 and discussion 384-392 does not make sense to me. Especially the following; “Although the immunoprecipitated POLA domain did not have detectable activity in a primer extension assay (Fig. S4), IC-POLA complementation indicated that the polymerase domain alone is sufficient to support minicircle replication even though IC-POLA localized mainly to the mitochondrial matrix (Fig. 4,5B ,C). IC-POLA includes all the conserved Family A motifs, but this truncated fragment may lack important upstream residues required for binding the primer template in the in vitro assay. Despite IC-POLA mislocalization during complementation, abundance was likely high enough to support the polymerase function.”

If the POLA domain does not have any primer extension activity how can it support minicircle replication? Can the authors please provide a more realistic hypothesis please?

3. Point number 2 is particularly pertinent when we consider that the UCR domain partially rescues IC-UTR RNAi defects. Please clarify how the UCR domain, which contains a structural portion of the protein, can partially rescue cells from a knockdown when in fact this domain does not seem to have any POLA or 3’-5’ exonuclease domains or activity? Perhaps a more intense bio-informatics analysis of the UCR domain is required. It would be more useful for the reader if the authors explain in more detail how they think the UCR domain partially rescues IC-UTR RNAi defects and propose a model for this.

4. Also, if; “abundance was likely high enough to support the polymerase function.” then shouldn’t the IC-DEAD that was expressed in high amounts rescue the POLC RNAi? This is because the DEAD mutations are in the POLA domain, leaving the UCR domain (present in abundance, upto 6 day based on Fig.2) to rescue, theoretically, POLC RNAi? Please Clarify.

5. Line 333 refers to sup data 4D-F, however there is either a mixup in figure numbers or some data missing. I think this data is in S4 but I’m not sure.

6. I suggest that the authors recheck and extensively rewrite the figure legends because they do not help much in understanding the data (high quality) that is presented.

Minor;

1. For the reader who is naïve in the ways of RNAi knockdown in trypanosomes, please explain in the manuscript, how targeting the 3'UTR induces knockdown of the gene of interest in the background of expression of a tagged full-length or truncated version of the gene, without knocking down the tagged versions?

2. lines 92 and 398 - references have different fonts to main text.

3. Line 117 - full stop needed.

4. Line 183-6 - does not make any sense, although I think I know what the meaning of the sentence is. Please rewrite it.

5. Line 205 and elsewhere – please check spaces between brackets, words etc, and for the absence of full stops etc.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes a detailed dissection of the function of the mitochondrial DNA polymerase POLIC in Trypanosoma brucei. This work is important because mechanisms of mitochondrial DNA replication and maintenance are of broad interest, and the multiplicity of replication proteins in kinetoplastids is an ongoing mystery. The approaches used are effective, the quality of the data is high, and their results generally support their conclusions excepting a few clarifications requested in my suggestions to authors. It clearly contributes to our mechanistic knowledge of the spatial and temporal regulation of kDNA replication as well as the link between the processes of replication and segregation, which has been suggested by other studies. The manuscript is-well written, although there are some changes that would make it more accessible to the reader.

Comments for the author

General comments:
For the Figures, it would be helpful if legends (for symbols, colors for different samples/categories) were shown next to the graphs themselves in addition to being described in the figure legends.

Based on the conclusions of the paper, I think that the wild-type rescue experiments (currently supplemental Fig S2) would work better as part of the main text. This is also the only opportunity where we get to see what the “normal” distribution of POLIC foci is during the cell cycle (Fig. S2F) without consulting previous literature. This is a useful point of comparison for similar analyses in the other figures.

Consistent naming of cell lines in figures and in the main text would be helpful. For instance, in Fig. 2 the graphs could be labeled OE dead and RNAi+OE dead so that it is clear what is being overexpressed in each case. This is done in Fig 3 and others but should be used throughout.

Figure 2E: I found these graphs confusing, especially without a reference to what the wild-type distribution of IC is. Moving Fig S2 from supplemental to main text will help with this. Also, I thought the Y-axis here would be more appropriately labeled “% of cells” or “% of cells with detectable POLIC” since it’s only the green striped portion of the bar that is showing PTP foci. This is especially true for Fig. 4E.

Complementation with the pol A domain only failed to fully rescue the phenotype, although it did seem to rescue the effect on free minicircles. However, the construct did not have the expected activity (Fig. S4). Could this explain the failure to rescue? Without this, can the authors conclude that the pol A domain is really not involved in segregation? Expressing a non-functional domain could actually have a dominant-negative effect which could explain the increase in asymmetric and ancillary kDNA?

As the authors state, it is a pleiotropic phenotype, and as I read I had a hard time keeping track of which complementation experiments rescued which defects. Perhaps the authors could include a table in which each row represents a complementation experiment and the columns indicate the effect on each phenotype: growth, foci formation, asymmetric kDNA, ancillary kDNA, and minicircles/CC:NG ratio. This might help them support their conclusions that different regions of the protein are responsible for different functions.

The authors suggest that POLIC is the clearest link to date between kDNA replication and segregation via the TAC, which may be true, but the authors might consider citing previous work on UMSBP, which showed defects in both replication and kDNA segregation (Milman et. al., 2007) and Sykes et. al., 2013, which describes another antipodal site protein that seems to play a role in kDNA distribution.

Specific comments:
In the legend for Fig 1A there appears to be some redundant text (Lines 808-810).

In the legend for Fig S2B, it says that the blue/white striped bars are “induced”, but doesn’t indicate if that refers to RNAi induced or RNAi +IC WT induced. Clearly from the results it’s the complementation result, but better labeling/nomenclature would make it easier on the reader.

According to lines 185-186, RNAi-mediated knockdown of POLIC using the 3’ UTR resulted in “no significant changes” in the abundance of transcripts from paralogs POL1B and POL1D. However, Fig 1B appears to show a statistically significant increase in the transcript for POL1D. This also occurs in other complementation experiments presented in the paper (Figs. 2B, 4B and S2B). Could the authors comment on this, perhaps in the Discussion? Might this be a compensatory response to a replication defect? The authors briefly mention “functional interplay” in lines 406-407 but I am surprised that this would happen at the transcript level, and it seems to be consistent across their experiments.

Lines 190-199: ancillary kDNA needs to be defined and that definition compared to asymmetric or fragmented kDNA. Also, was ancillary kDNA observed in the 2002 paper and just not quantified as a separate category? In general, I found the description of these two “stages” of quantitation to be a little confusing. It should be fleshed out to make it clearer what was counted in each case (and how the categories relate to each other, i.e would “ancillary” and “asymmetric” in the second stage both be included in the “fragmented/asymmetric category” of the first stage?). Alternatively, just the second stage of quantitation (that shown in Fig 1D) could be shown and discussed. I think it’s still clear that the phenotype resembles that described in Klingbeil et. al., 2002 and the updated categories are the ones used for the rest of the paper.

Lines 188-191: this description should be moved to the Methods. I think it’s sufficient to say that “parasites per time point were classified according to kDNA size and distribution” or similar.

Lines 256-257: I was confused by the statement that the “IC-DEAD complementation depleted endogenous POLIC transcripts, while abundance of this variant was significantly higher than endogenous POLIC”. Surely it’s the RNAi mediated by the UTR dsRNA that is depleting endogenous IC transcripts, and the variant is higher because it’s being overexpressed? The way it’s written it sounds like there is some kind of complex interaction between the expressed variant and the abundance of the endogenous transcript.

Reviewer 3

Advance summary and potential significance to field

Authors in this manuscript dissect the function of the two structural domains of POL1C, which is one of the three major DNA polymerases; POL1B POL1C, and POL1D, involved in kDNA replication. Studies presented here are significant because understanding the function of different structural domains of POL1C would help to define the role of this polymerase in the unique replication process of kDNA in T. brucei. Authors demonstrated that overexpression of the wild type POL1C could rescue the RNAi phenotype, but the active site mutant of POL1C showed a dominant negative phenotype for the loss of function. Next they tested the ability of the two structural domains of POL1C, 1) the C-terminal DNA Polymerase A (POLA) domain and the 2) N-terminal uncharacterized region (UCR) for their ability to complement the RNAi phenotype. Their results showed that overexpression of the POLA domain partly rescue the growth and replication (kDNA) defect of the RNAi cells but failed to form cell cycle-dependent foci at the anti-podal sites. On the other hand, overexpression of the UCR region showed foci formation similar to the full-length wild type POL1C, but didnÂ’t complement the growth and replication defect due to POL1C RNAi, indicating that the UCR region is essential for specific localization of this enzyme during cell cycle and polymerase activity is needed for kDNA replication. Authors suggested that antipodal sites foci formation is dispensable due to overexpression of the POLA.

Comments for the author

Authors in this manuscript dissect the function of the two structural domains of POL1C, which is one of the three major DNA polymerases; POL1B POL1C, and POL1D, involved in kDNA replication. Studies presented here are significant because understanding the function of different structural domains of POL1C would help to define the role of this polymerase in the unique replication process of kDNA in T. brucei. Authors demonstrated that overexpression of the wild type POL1C could rescue the RNAi phenotype, but the active site mutant of POL1C showed a dominant negative phenotype for the loss of function. Next they tested the ability of the two structural domains of POL1C, 1) the C-terminal DNA Polymerase A (POLA) domain and the 2) N-terminal uncharacterized region (UCR) for their ability to complement the RNAi phenotype. Their results showed that overexpression of the POLA domain partly rescues the growth and replication (kDNA) defect of the RNAi cells but failed to form cell cycle-dependent foci at the anti-podal sites. On the other hand, overexpression of the UCR region showed foci formation similar to the full-length wild type POL1C, but didn’t complement the growth and replication defect due to POL1C RNAi, indicating that the UCR region is essential for specific localization of this enzyme during cell cycle and polymerase activity is needed for kDNA replication. Authors suggested that antipodal sites foci formation is dispensable due to overexpression of the POLA. Data presented here are clean and interesting, however, the role of the UCR region on kDNA segregation is not convincing. Additional experiments are needed to support that POL1C has dual role in kDNA replication and segregation. Few additional comments and questions are listed below;

1. What is the level of interdependency of POL1C and POL1D for their function and localization? Author’s previous work reported that POL1D knockdown reduced the levels of POL1D and POL1C transcripts. In this manuscript, POL1C RNAi showed some effect on POL1D transcript (Fig. 1B). It would be interesting to know the effect of individual RNAi on the protein levels of other polymerases. Do the polymerases interact with each other?

2. POL1C-DEAD expression was greatly reduced after day 6 of induction however, the growth defect was found mostly after day 6 (Fig. 2). How this could be explained.

3. Fig. 5A and D, legends for Y axis are missing.

4. Over expression of POLA domain minimally changed the number of cells with small kDNA and other abnormal kDNAs in comparison to the RNAi cells.
Therefore it should be stated that POLA domain partially rescues the minicircle replication defect.

5. Additional EM data are needed to show if POL1C plays role in kDNA segregation.

First revision

Author response to reviewers' comments

November 7, 2019

Re: Manuscript ID#: JOCES/2019/233072

Dear Dr. Glover,

We have carefully reviewed the referees' comments on our manuscript **"Structure-function analysis reveals a DNA polymerization-independent role for mitochondrial DNA polymerase IC in African trypanosomes"** and we thank the reviewers for their time and supportive comments about our work. We also thank the reviewers for their critical analysis of our manuscript.

We feel that the Reviewers' comments have improved our submission and have helped to clarify points that make this an even stronger submission. We have addressed each comment by the Editor and the reviewers (see below). Our responses are highlighted in **blue (below) and modifications to the manuscript are highlighted with yellow.** Additionally, we have included the line number in the manuscript for quick reference to read where changes have been made.

Journal Requirements:
1. Please read our requirements for preparing your figures (jcs_revision.pdf, attached) to avoid a potential delay in the publication process or rejection on the basis of non-compliance with these guidelines.

We have carefully checked all of the figures and made any necessary corrections as recommended by the Journal guidelines.

2. The length limit for Research Articles is 8000 words, and 3000 for Short Reports.

After incorporating the reviewers' suggestions the word count for the manuscript is 7194.

3. Please note that the original source files for text, tables and all figures will be required.

We are adhering to the Journal guidelines for all tables, figures and the text.

4. Supplementary figures and tables to be submitted as one PDF file, including figure legends.

We have combined all the supplemental material into a single PDF file.

5. Authors are requested to fill in and upload a submission checklist with their manuscript.

We have addressed all items in the checklist and uploaded the PDF file.

6. Adherence to a strict limit for the title of the paper to be under 120 characters, including spaces.

We have changed the title to now read “A DNA polymerization-independent role for mitochondrial DNA polymerase IC in African trypanosomes” bringing the character count down to 97.

7. Include a list of symbols and abbreviations.

The list of symbols and abbreviations can be found right before the references.

Reviewer 1:

1. Line 209 211 and other places, the authors state that; “Taken together, IC-UTR silencing phenocopied the previously reported LOF, and kDNA replication defect but also displayed clear signatures of a segregation defect”. Implying that knockdown of POLC disturbs kinetoplast segregation. This is incorrect because the authors did not interfere with the kinetoplast segregation machinery per se, which, as they mention, is a basal body based segregation machinery. Unless, they have basal body/flagella markers or measurements to prove this. From the data they have provided in the manuscript, in my humble opinion, they have interfered with the structure or component of the network that is attached to the segregation machinery. Thus, ancillary or components of kinetoplasts as well as marker proteins such as TAC 102 are floating around in the mitochondrion matrix. Can they please rethink the text and find a replacement for their idea of segregation with something else? Alternatively provide evidence that the segregation machinery is affected by POLIC knockdown.

We agree with the humble opinion of reviewer 1 and have thought deeply about the terminology to be used to describe the non-replicative aspect of the pleiotropic phenotype. We agree that segregation is not an accurate description of the phenotype and have therefore substituted segregation with “distribution”. (see numerous places where distribution has now replaced segregation). With the accumulation of more pleiotropic RNAi defects in our field, we have taken this opportunity to more precisely define the intermediary stage between replication and segregation (Line 162-165).

We have rephrased the last two lines in the abstract to reflect use of the new term distribution (Lines 42-44).

Additionally, we have added text to the introduction to clearly define distribution and when it would be occurring in relationship to the other kDNA associated transactions (Lines 97-98; 160-165).

2. Results in lines 319-320 and discussion 384-392 does not make sense to me. Especially the following; “Although the immunoprecipitated POLA domain did not have detectable activity in a primer extension assay (Fig. S4), IC-POLA complementation indicated that the polymerase domain alone is sufficient to support minicircle replication even though IC-POLA localized mainly to the mitochondrial matrix (Fig. 4,5B ,C). IC-POLA includes all the conserved Family A motifs, but this truncated fragment may lack important upstream residues required for binding the primer template in the in vitro assay. Despite IC-POLA mislocalization during

complementation, abundance was likely high enough to support the polymerase function. ”If the POLA domain does not have any primer extension activity how can it support minicircle replication? Can the authors please provide a more realistic hypothesis please?

We agree that it is unusual that activity was not detected *in vitro*. However, our molecular data (Southern blot of free minicircles) clearly indicate that complementation with IC-POLA domain alone can partially rescue replication defects based on: 1. Ratio of unreplicated (CC) to replicated (N/G) free minicircles remains unchanged during IC-POLA complementation and 2. The abundance of free minicircles does not increase as in the parental RNAi cell line (IC-UTR). Additionally, the IC-POLA domain is detected near the kDNA in addition to the matrix localization. This is stated in the results section (Lines 317-318).

We hypothesize that *in vivo*, the IC-POLA variant may have access to other accessory factors that allow it to support maintenance of free minicircles.

We clarify in the discussion that weak signal was detected around the kDNA disk instead of describing the localization as generally “mislocalized” as we previously wrote in the discussion, and provided text highlighting the hypothesis that accessory factors could be supporting the maintenance of free minicircles (Lines 410-414).

3. Point number 2 is particularly pertinent when we consider that the UCR domain partially rescues IC-UTR RNAi defects. Please clarify how the UCR domain, which contains a structural portion of the protein, can partially rescue cells from a knockdown when in fact this domain does not seem to have any POLA or 3’-5’ exonuclease domains or activity? Perhaps a more intense bio-informatics analysis of the UCR domain is required. It would be more useful for the reader if the authors explain in more detail how they think the UCR domain partially rescues IC-UTR RNAi defects and propose a model for this.

We applied several bioinformatics analyses to gain more information on the UCR including PSI pred, CFSSP, YASPIN, and InterPRO using default parameters. At this time, we have not performed a more extensive bioinformatics analysis. However, there are several proteins that localize to the AS and including them in a more extensive search may provide a common motif that can be experimentally verified as targeting to the AS.

We have added text to Materials and Methods to provide an explanation for choosing truncation sites based on secondary structure prediction (Lines 521-524).

Additionally, in the results section and discussion we highlight that the UCR does contain all 3 arginine methylation sites identified in a mitochondrial methylarginine proteome study (Fisk et al, 2011) (Lines 336-338, 380-385).

To address a model on how the UCR may be functioning, we previously mentioned that spatiotemporal localization likely contributes to the dual roles of POLIC (Lines 443-445). We have added text that highlights a potential role for arginine methylation where the methylation status of the protein may dictate one of the functions or possibly even localization. (Lines 448- 453).

4. Also, if; “abundance was likely high enough to support the polymerase function.” then shouldn’t the IC-DEAD that was expressed in high amounts rescue the POLC RNAi? This is because the DEAD mutations are in the POLA domain, leaving the UCR domain (present in abundance, upto 6 day based on Fig.2) to rescue, theoretically, POLC RNAi? Please Clarify.

We understand the reviewer’s question as follows: shouldn’t we expect IC-DEAD complementation to at least rescue the distribution defect because the UCR is present and only the POLA domain is mutated. Additionally, it seems that the reviewer is linking the previous abundance argument of the IC-POLA domain to the IC-DEAD variant.

First, we have replaced in the discussion (Lines 410-414) the previous IC-POLA abundance statement with a hypothesis that relates to the localization of the IC-POLA domain (see response to reviewer 1 comment 2 above). Specifically, *in vivo*, the IC-POLA variant may have

access to other accessory factors that allow it to support maintenance of free minicircles.

Based on the spatiotemporal dynamics of kDNA replication, and the previously reported POLIC cell cycle localization, IC (or the variants) likely first performs in a replication function prior to any distribution function. If there is a strong replication defect, the amount of kDNA material continuously declines as we observed with IC-DEAD (OE and Comp), indicating that there was a dominant negative defect associated with inactivating the POLA domain.

We agree with the reviewer that overexpression of a variant with a functional UCR domain should theoretically rescue the distribution defect. In fact, IC-DEAD comp showed no asymmetric networks (see Fig 8). We had added text to the results to point out that no asymmetric networks were observed with IC-DEAD comp (Lines 284-285).

However, we did not put a lot of emphasis on a distribution rescue since the population lost kDNA so rapidly. It is possible that we did not observe asymmetric networks solely because the replication defect would supersede seeing a distribution defect in this case.

5. Line 333 refers to sup data 4D-F, however there is either a mixup in figure numbers or some data missing. I think this data is in S4 but I'm not sure.

We apologize for this confusion. This should have been referencing the IC-UCR overexpression supplemental data. With the move of Fig S2 to the main text, the overexpression data is now Fig S4 so the text will remain the same (Line 343).

6. I suggest that the authors recheck and extensively rewrite the figure legends because they do not help much in understanding the data (high quality) that is presented.

We have rewritten figure legends to clarify the data and made them more concise in some instances.

7. For the reader who is naïve in the ways of RNAi knockdown in trypanosomes, please explain in the manuscript, how targeting the 3'UTR induces knockdown of the gene of interest in the background of expression of a tagged full-length or truncated version of the gene, without knocking down the tagged versions?

We have added text to explain that the variants are expressed with a different 3' UTR that makes them resistant to POLIC 3'UTR mediated gene silencing (Lines 184-187, and Lines 225-226).

8. Lines 92 and 398 - references have different fonts to main text.

Thank you for pointing this out – these are corrected on lines 92 and 402 respectively.

9. Line 117 - full stop needed.

Missing full stop has been added (Line 118).

10. Line 183-6 - does not make any sense, although I think I know what the meaning of the sentence is. Please rewrite it.

This sentence has been reworded to correctly describe the data (Lines 192-194).

11. Line 205 and elsewhere – please check spaces between brackets, words etc, and for the absence of full stops etc.

Thank you for pointing out these typos. We have carefully gone through the manuscript and addressed these issues, however we have not highlighted each corrected instance.

Reviewer 2:

1. For the Figures, it would be helpful if legends (for symbols, colors for different samples/categories) were shown next to the graphs themselves in addition to being described in the figure legends.

We agree and have added legends to graphs that contain more than two datasets, which include kDNA quantifications and the nucleotide incorporation assay.

2. Based on the conclusions of the paper, I think that the wild-type rescue experiments (currently supplemental Fig S2) would work better as part of the main text. This is also the only opportunity where we get to see what the "normal" distribution of POLIC foci is during the cell cycle (Fig. S2F) without consulting previous literature. This is a useful point of comparison for similar analyses in the other figures.

We agree. Supplemental Figure 2 is now Figure 2 in the main text to provide an easier comparison. We have adjusted the text accordingly. Reference to this new figure can be found on lines 223-242.

3. Consistent naming of cell lines in figures and in the main text would be helpful. For instance, in Fig. 2 the graphs could be labeled OE dead and RNAi+OE dead so that it is clear what is being overexpressed in each case. This is done in Fig 3 and others but should be used throughout.

Thank you for pointing out the inconsistency with our nomenclature of the cell lines. We have added specificity to indicate which variant is being analyzed/described. These corrections are made throughout the text and within each of the figures.

4. Figure 2E: I found these graphs confusing, especially without a reference to what the wild-type distribution of IC is. Moving Fig S2 from supplemental to main text will help with this. Also, I thought the Y-axis here would be more appropriately labeled "% of cells" or "% of cells with detectable POLIC" since it's only the green striped portion of the bar that is showing PTP foci. This is especially true for Fig. 4E.

As indicated above, we have moved Fig S2 to the main text so this comment is now addressing new Figure 3. We have changed the Y-axis to read "Percent of cells".

5. Complementation with the pol A domain only failed to fully rescue the phenotype, although it did seem to rescue the effect on free minicircles. However, the construct did not have the expected activity (Fig. S4). Could this explain the failure to rescue? Without this, can the authors conclude that the pol A domain is really not involved in segregation? Expressing a non-functional domain could actually have a dominant-negative effect which could explain the increase in asymmetric and ancillary kDNA?

Thank you for highlighting this nuance. We edited the Results section for the POLA variant to describe a "partial rescue" for kDNA replication due to the restoration of free minicircles (Line 304). In response to reviewer 1's comment 2, we clarified possible reasons for IC-POLAs lack of activity *in vitro* but partial rescue *in vivo*.

As indicated in reviewer 1 comment 1, the other function that we are attributing to IC is no longer segregation but a distribution role.

We must now address whether we can conclusively state that POLA has no function in segregation (which we have now clarified as distribution in Lines 162-165). We apologize that the reviewer was left with this impression. In the discussion (Line 467-468) we did state that the POLA domain may be involved in distribution.

We have taken this opportunity to better clarify roles of the individual variants by including a new summary figure, Figure 8 that reviewer 2 suggests in comment 6. See comment below.

Lastly, we agree with the reviewer that expressing a non-functional domain could lead to a

dominant negative effect. However, we never saw loss of fitness, persistent changes to free minicircles or kDNA morphology with just overexpressing the POLA domain (Fig 5A, Fig S4 B, C).

6. As the authors state, it is a pleiotropic phenotype, and as I read I had a hard time keeping track of which complementation experiments rescued which defects. Perhaps the authors could include a table in which each row represents a complementation experiment and the columns indicate the effect on each phenotype: growth, foci formation, asymmetric kDNA, ancillary kDNA, and minicircles/CC:NG ratio. This might help them support their conclusions that different regions of the protein are responsible for different functions.

We totally agree with the reviewer and have composed a summary figure (Fig 8) highlighting the comparative defects and thank the reviewer for this great suggestion. Compiling the data in a single table greatly facilitated the recognition of the distribution defect, namely the accumulation of asymmetric networks when the UCR domain was absent.

We have added text that refers to Fig 8 at the end of results section (Lines 358-359) and have incorporated additional text referring to Fig 8 in the discussion (Lines 419, 459, 463, 469).

7. The authors suggest that POLIC is the clearest link to date between kDNA replication and segregation via the TAC, which may be true, but the authors might consider citing previous work on UMSBP, which showed defects in both replication and kDNA segregation (Milman et. al., 2007) and Sykes et. al., 2013, which describes another antipodal site protein that seems to play a role in kDNA distribution.

We thank the reviewer for reminding us of these important references. We had not included them because the POLIC phenotype was distinct from depletion of either USMBP or α-KDE2. These two references are good examples of other dual-functioning kDNA related proteins and should be included in the discussion.

We have added text to compare/ contrast POLIC defects with those of UMSBP and α-KDE2. In this same paragraph, we changed the statement about POLIC providing direct evidence for an interaction between replication and segregation machineries (lines 380-381 in previous version) to state that POLIC is the clearest example bridging the replication and segregation processes at this time (Lines 395-403).

8. In the legend for Fig 1A there appears to be some redundant text (Lines 808-810).

Thank you, we have removed the redundant text from the figure legend (Lines 901-903).

9. In the legend for Fig S2B, it says that the blue/white striped bars are “induced”, but doesn’t indicate if that refers to RNAi induced or RNAi +IC WT induced. Clearly from the results it’s the complementation result, but better labeling/nomenclature would make it easier on the reader.

The comment refers to the new Figure 2 and, we have added the identifier RNAi+OE IC-WT to make it easier to understand what the experiment is.

10. According to lines 185-186, RNAi-mediated knockdown of POLIC using the 3’ UTR resulted in “no significant changes” in the abundance of transcripts from paralogs POL1B and POL1D. However, Fig 1B appears to show a statistically significant increase in the transcript for POL1D. This also occurs in other complementation experiments presented in the paper (Figs. 2B, 4B and S2B). Could the authors comment on this, perhaps in the Discussion? Might this be a compensatory response to a replication defect? The authors briefly mention “functional interplay” in lines 406-407 but I am surprised that this would happen at the transcript level, and it seems to be consistent across their experiments.

The primary objective of evaluating relative mRNA of related kDNA pols (POLIB, POLID) was to show that they are not inadvertently targeted during POLIC depletion. The original statement was meant to reflect the idea that there was no statistically significant decline in transcript of

the related kDNA pols. We have corrected the text to clarify (Lines 192-194).

It is important to point out that gene expression regulation in trypanosomes is almost exclusively post- transcriptional. So, changes at the transcript level do not always reflect protein abundance (Clayton 2016, Curr Opin Micro 32:45-51).

Yes, the changes in transcript levels are likely to be a response to the replication defect, but by an unknown mechanism at this time. We have data from dual gene silencing experiment (POLIB/POLID) that suggest a complex retrograde response in triggered when kDNA is depleted rapidly and completely. However, this is unpublished data.

The biological significance for slight upregulation for *POLIB* and *POLID* transcripts observed in most experiments is not known. We agree that functional interplay at the transcript level would be unlikely and have therefore removed the statement.

Instead, we suggest that IC-DEAD may be inhibiting kDNA access for POLID or other replication proteins and included reference to our previous data that also suggest some type of interaction (physical or functional) among the paralogs (Lines 432-436).

11. Lines 190-199: ancillary kDNA needs to be defined and that definition compared to asymmetric or fragmented kDNA. Also, was ancillary kDNA observed in the 2002 paper and just not quantified as a separate category? In general, I found the description of these two "stages" of quantitation to be a little confusing. It should be fleshed out to make it clearer what was counted in each case (and how the categories relate to each other, i.e would "ancillary" and "asymmetric" in the second stage both be included in the "fragmented/asymmetric category" of the first stage?). Alternatively, just the second stage of quantitation (that shown in Fig 1D) could be shown and discussed. I think it's still clear that the phenotype resembles that described in Klingbeil et. al., 2002 and the updated categories are the ones used for the rest of the paper.

We thank the reviewer for pointing out that we did not define ancillary in our document. We added text (Line 156-157) to define ancillary kDNA. Asymmetric (Line 202) and fragmented networks (Lines 182-183) were also defined. Ancillary kDNA was only observed in the 2002 paper and not quantified and the justification for why it was important to distinguish and quantify these categories was also clarified (Lines 201-208). We chose to not change Figure 1 but to include clarification of the different categories.

12. Lines 188-191: this description should be moved to the Methods. I think it's sufficient to say that "parasites per time point were classified according to kDNA size and distribution" or similar.

This description was moved to the methods and has been replaced with the suggested succinct description (Lines 196-197).

13. Lines 256-257: I was confused by the statement that the "IC-DEAD complementation depleted endogenous POLIC transcripts, while abundance of this variant was significantly higher than endogenous POLIC". Surely it's the RNAi mediated by the UTR dsRNA that is depleting endogenous IC transcripts, and the variant is higher because it's being overexpressed? The way it's written it sounds like there is some kind of complex interaction between the expressed variant and the abundance of the endogenous transcript.

We added additional detail to better describe the genetic complementation system in response to Reviewer 1 comment 7 (see above).

We apologize for the confusing wording surrounding the genetic complementation system. To clarify, we have added text that states "In this genetic complementation system, the tagged POLIC variant utilizes an unrelated 3'UTR to avoid targeting by RNAi (Line 225-226). This should make it clear that the RNAi targets the endogenous POLIC UTR, while the variant contains an unrelated UTR to avoid targeting altogether.

Additionally, we have simplified the confusing text that the reviewer identified. The text now states RT- qPCR confirmed depletion of endogenous *POLIC* transcripts, and increased abundance of the variant transcript (Lines 268-269).

Reviewer 3:

1. What is the level of interdependency of POL1C and POL1D for their function and localization? Author's previous work reported that POL1D knockdown reduced the levels of POL1D and POL1C transcripts. In this manuscript, POL1C RNAi showed some effect on POL1D transcript (Fig. 1B). It would be interesting to know the effect of individual RNAi on the protein levels of other polymerases. Do the polymerases interact with each other?

We agree with the Reviewer that it would be interesting to know if the protein levels of the other DNA polymerases are impacted when each of the paralogs are silenced and whether the DNA polymerases interact with one another. We also appreciate the reviewer's interest in our previous work.

Our previous work used a cell line in which *POLIC* was endogenously tagged and that allowed *POLID* RNAi. When *POLID* was depleted there was a 55% decline in *POLID* transcripts while *POLIC* transcripts showed only a minor decline of about 15% that was accompanied by a statistically significant decrease in POLIC-PTP at the end of the induction and mislocalization of the remaining POLIC-PTP (no antipodal site foci formation). At this time, we do not know if there is interdependence between POLIC and POLID for their functions and localization, or if the results reflected more global defects.

Additionally, we do not have reliable specific antibodies to test for these putative interactions. In the absence of specific antibodies it would require generating separate cell lines for each of the circumstances and for co-IP experiments. Unpublished data on separate tandem affinity purifications of PTP-tagged POLIB, POLIC, and POLID indicate that the other paralogs were not detected when the final eluates were analyzed via Mass spectrometry. Transient interactions among the DNA pols might also explain this data.

Our current study with the POLIC variants has established distinct aspects of kDNA distribution that could impact the localization and function of POLIB and POLID. We feel that this information is a strong foundation to now move forward and study potential interactions; physical or functional. However at this time we feel that those types of experiments are beyond the scope of this study.

2. POL1C-DEAD expression was greatly reduced after day 6 of induction, however, the growth defect was found mostly after day 6 (Fig. 2). How this could be explained.

We are not certain why the protein abundance of IC-DEAD declines after 6 days of induction, but this was consistent on three separate inductions for both overexpression and complementation.

The reviewer is correct that loss of fitness is most notable around Day 6 in both the overexpression and complementation experiments. However, a decrease in fitness begins as early as Day 4 when IC- DEAD levels are higher. The delay in growth inhibition following loss of kDNA is typical for many kDNA replication defects in which defects at the molecular level are detected before loss of fitness at the cellular level. This is likely due to the time required for the minicircles that encode essential guide RNAs to be depleted below a critical threshold (Bruhn et al 2010, Wang et al 2002).

In the case of IC-DEAD, there is notable accumulation of small kDNA (36%- OE, 26%-Comp) as early as Day 2 of induction. By Day 4, there is continued increase in the % of cells with small kDNA (43%- OE, 58%-Comp). Additionally, the % of cells with no kDNA increases (7%-OE, 20%-Comp). This is exactly when the loss of fitness is first observed likely correlating with crossing the threshold for loss of minicircles that encode essential gRNAs. The levels of IC-DEAD variant do not need to be expressed throughout the induction. Once the minicircles with the essential gRNAs are lost, there would be no way to recover the lost minicircles.

3. Fig. 5A and D, legends for Y axis are missing.

Thank you for pointing this out – we have corrected this in the new Figure 6.

4. Over expression of POLA domain minimally changed the number of cells with small kDNA and other abnormal kDNAs in comparison to the RNAi cells. Therefore it should be stated that POLA domain partially rescues the minicircle replication defect.

We believe that this reviewer is referring to the title of the results section for the POLA variant (Line 304). We have changed the title to reflect that indeed it is only a partial rescue. We have also changed and added content to the discussion based on comments from reviewer 1, comment 2 and reviewer 2 comment 5.

5. Additional EM data are needed to show if POL1C plays role in kDNA segregation.

Based on the comments from reviewers, we have changed the terminology of the additional role for POLIC from segregation to distribution of the kDNA network. Please refer to Reviewer 1 comment 1 and Reviewer 2 comment 5 and comment 7 for details. Therefore, we feel at this time EM data are not necessary to support the conclusions of the manuscript.

Sincerely,

Michele Klingbeil, Ph.D.
Associate Professor of Microbiology

Second decision letter

MS ID#: JOCES/2019/233072

MS TITLE: A DNA polymerization-independent role for mitochondrial DNA polymerase IC in African trypanosomes

AUTHORS: Jonathan C Miller, Stephanie B Delzell, Jeniffer Concepcion-Acevedo, Michael J Boucher, and Michele M Klingbeil
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper is now clear and has more focused orientation towards the subject matter. I am not sure if changing the word "segregation" to "distribution" is sufficient because these words have similar definitions but overall the written modifications are well done and adequate. For me this paper is now suitable for publication in J. Cell. Sci.

Comments for the author

Comment; I am still having difficulties with the precision in some parts of this manuscript. For example line 51-53 states “Despite decades of investigation, the mechanisms that regulate replication, copy number maintenance and segregation of mtDNA remain unknown.“ This is not true. The mechanism that regulates segregation is known.

The Merriam-Webster dictionary definitions for segregate and distribution are;
Distribute; To divide or separate especially into kinds.
Segregate; To separate or set apart from others or from the general mass.

Without trying to be pedantic on my part it is clear that changing from segregate to distribution does not change the meaning of the phrase. Indeed, the phenotype is not related to segregation or distribution. The segregation machinery is working. The problem is the link between the kDNA to that machinery. Proof is that in Line 282. kDNA segregation is normal in IC-DEAD complementation experiments. Also, Line 344. kDNA segregation is normal in IC-UCR complementation. Therefore, line 470 is correct. It is likely that POLIC is not playing a direct role in distribution but is impacting other proteins essential for distribution.

Minor

Fig 3A shows growth curves but in the text (line 281) it reads; In striking contrast to both OE and the parental RNAi phenotypes, IC-DEAD complementation displayed a majority of cells with no kDNA (59.1%) that correlated with the exacerbated LOF. IC-DEAD also displayed small (16.1%), ancillary (10.8%), other (9.6%), and normal (4.4%) kDNA, but did not display any asymmetric kDNA networks (Fig 3D). There is a mix-up?

In summary however, this paper is now suitable for publication in J. Cell. Sci.

Reviewer 2

Advance summary and potential significance to field

This work represents a thorough mechanistic dissection of a novel DNA replication protein that plays multiple roles in kDNA replication and distribution in Trypanosoma brucei.

Comments for the author

The authors have addressed all of my concerns in the revised manuscript, which I find to be much improved. I have no further comments.

Second revision

Author response to reviewers' comments

January 28, 2020

Re: Manuscript ID#: JOCES/2019/233072

Dear Dr. Glover,

We feel that all of the Reviewers' comments (from both rounds of reviewing) have improved our submission and have helped to clarify points that make our manuscript an even stronger submission. We were especially encouraged that both reviewers agree that our work is "now suitable for publication" in the Journal of Cell Science. Reviewer 2 did not have any further comments and felt that our work represented "a thorough mechanistic dissection of a novel DNA replication protein." However, Reviewer 1 had a few remaining comments that we address below.

Our responses are highlighted in **blue (below) and modifications to the manuscript are highlighted with green.** We have retained the original yellow highlighted changes for reference. Additionally, we have included the line number in the manuscript for quick reference to read where changes have been made.

Journal Requirements:
1. Please read our requirements for preparing your figures (jcs_revision.pdf, attached) to avoid a potential delay in the publication process or rejection on the basis of non-compliance with these guidelines.

We have prepared figures according to the Journal guidelines.

2. The length limit for Research Articles is 8000 words, and 3000 for Short Reports.

After incorporating the reviewers' suggestions the word count for the manuscript is 7249.

3. Please note that the original source files for text, tables and all figures will be required.

We are adhering to the Journal guidelines for all tables, figures and the text.

4. Supplementary figures and tables to be submitted as one PDF file, including figure legends.

We have combined all the supplemental material into a single PDF file.

5. Authors are requested to fill in and upload a submission checklist with their manuscript.

We have addressed all items in the checklist and uploaded the PDF file.

6. Adherence to a strict limit for the title of the paper to be under 120 characters, including spaces.

We changed the title in the previous version to bring the character count down to 97.

7. Include a list of symbols and abbreviations.

The list of symbols and abbreviations can be found right before the references.

Reviewer 1:

1. I am still having difficulties with the precision in some parts of this manuscript. For example line 51- 53 states “Despite decades of investigation, the mechanisms that regulate replication, copy number maintenance and segregation of mtDNA remain unknown.“ This is not true. The mechanism that regulates segregation is known.

We agree with the reviewer that the concluding sentence (line 51-53) needed to be more precise. The paragraph was related to biomedical research and we were implying that the regulation for the mechanisms of those processes is not clearly defined for mammals. We have changed that concluding sentence to better reflect this. We also modified a sentence in the following paragraph (line 55) for better reading.

2. Without trying to be pedantic on my part it is clear that changing from segregate to distribution does not change the meaning of the phrase. Indeed, the phenotype is not related to segregation or distribution. The segregation machinery is working. The problem is the link between the kDNA to that machinery. Proof is that in Line 282. kDNA segregation is normal in IC-DEAD complementation experiments.
Also, Line 344. kDNA segregation is normal in IC-UCR complementation. Therefore, line 470 is correct. It is likely that POLIC is not playing a direct role in distribution but is impacting other proteins essential for distribution.

While complementation with IC-DEAD does show a reduction in asymmetric networks, ancillary kDNA is not dramatically reduced compared to the IC-UTR RNAi parental cell line. In a mixed phenotype where there is dramatic loss of kDNA, it is challenging to know whether segregation is proceeding normally.

Povelones (2014) makes an argument that defects in asymmetrical division could be masked by rapid loss of the kDNA. However, the argument did not address how the unusual ancillary kDNA phenotype might arise.

Our response is similar regarding the IC-UCR complementation. There is a reduction in asymmetric networks, but no change in ancillary kDNA compared to the IC-UTR parental RNAi cell line. At this time the mechanism of how ancillary kDNA is generated is unknown as several proteins with no direct role in kDNA replication also display the ancillary kDNA phenotype.

We hesitate to describe the POLIC depletion phenotype as linking the kDNA to the segregation machinery because we have no data that strongly supports that claim, and POLIC may have a secondary or indirect impact on linking the kDNA to the segregation machinery as we state in line 470.

3. Fig 3A shows growth curves but in the text (line 281) it reads; In striking contrast to both OE and the parental RNAi phenotypes, IC-DEAD complementation displayed a majority of cells with no kDNA (59.1%) that correlated with the exacerbated LOF. IC-DEAD also displayed small (16.1%), ancillary (10.8%), other (9.6%), and normal (4.4%) kDNA, but did not display any asymmetric kDNA networks (Fig 3D). There is a mix-up?

Yes this indeed was a mix-up. We thank the reviewer for catching this error before publication. We corrected the figure reference to read Fig 4A (line 281).

Based on the numerous changes that were made in the last version, we also checked reference to other figures throughout the manuscript and discovered one additional “mix-up”. We corrected figure reference on line 351 to now read Fig 6D.

Reviewer 2:

We thank the reviewer for recognizing our efforts to address the numerous reviewer's comments and improve upon our original submission.

Sincerely,

Michele Klingbeil, Ph.D.
Associate Professor of Microbiology

Third decision letter

MS ID#: JOCES/2019/233072

MS TITLE: A DNA polymerization-independent role for mitochondrial DNA polymerase IC in African trypanosomes

AUTHORS: Jonathan C Miller, Stephanie B Delzell, Jeniffer Concepcion-Acevedo, Michael J Boucher, and Michele M Klingbeil
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.