nature portfolio

# Peer Review File

# Stabilization of expandable DNA repeats by the replication factor Mcm10 promotes cell viability

Corresponding Author: Professor Sergei Mirkin

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study by Masnovo and coworkers describes an in-depth analysis of the role of Mcm10 in GAA repeat stability in budding yeast. The hypomorphic mcm10-1 mutation causes a more than 30-fold increase in GAA repeat expansions, and a milder 10-fold increase in contractions at the semi-permissive temperature of 27oC. The authors show convincingly that this effect is not due to the degradation of the catalytic subunit of DNA polymerase-alpha. Interestingly, deletion of Ctf4, a binding partner of Mcm10, has no impact on GAA expansion. The effect of mcm10-1 can be reversed by the mcm2-G400D suppressor mutation that alters the Mcm2 subunit of the CMG helicase. This strongly suggests that Mcm10's role in GAA repeat stability has something to do with its function during elongation and is independent of origin firing. Although the authors do not formally exclude that an origin firing defect is not responsible for the phenotype, they show very elegantly that Mcm10 regulates fork speed as the replisome passes through a (GAA)100 repeat cassette. Whereas forks move more slowly through the genome in mcm10-1 mutants, they don't slow down in GAA repeats. Together with the observation that overexpression of RPA in the mcm10-1 mutant background doesn't affect repeat expansion but rescues cell viability, the authors conclude that mcm10-1 accumulates single-stranded DNA and likely leads to RPA exhaustion. This is further corroborated by the finding that both DNA damage tolerance and S phase checkpoint activation promote cell viability at semi-permissive conditions. Exo1 doesn't have a role in expansion, but deletion partially rescues viability, arguing that the gaps generated in mcm10-1 mutants are exonucleolytically processed. All experiments are well controlled and convincing. They fully support the authors claims.

I have a minor comment about the model in Fig. 7C. I agree with the authors that single-stranded DNA likely results from DNA polymerase slippage. This could be directly tested by testing a pol-delta mutant with reduced processivity.

There are over 50 human disorders caused by repeat expansions, and this study is the first to demonstrate that Mcm10 modulates replication fork speed through GAA repeats. Mcm10's role in elongation has been highly controversial and this study does not only provide mechanistic insight into GAA repeat expansion but puts a long-standing controversy in the replication field to rest. The results are consistent with other reports demonstrating that human MCM10 controls replication through telomeric repeats and has a direct role in suppressing single-stranded gaps, making it plausible that MCM10 might have a similar role in the control of GAA repeats in human cells.

Reviewer #2

(Remarks to the Author)
The manuscript by Masnovo et al. explores the role of the replication factor Mcm10 in the genetic instability of GAA repeats using budding yeast as a model system.
It mainly employs a well-established system from the Mirkin lab for measuring the rate of repeat contractions or expansions, testing various double mutants to try and establish the mechanism by which Mcm10 affects repeat instability. In addition, they authors find that Mcm10 plays an important part in viability of yeast cells when expanded GAA repeats are located on an essential chromosome arm, and also define the genetic requirements for this role. Finally, the authors use live cell imaging to measure fork speeds through expanded GAA repeats in WT vs Mcm10 mutated cells.
The topic and overall experimental design and presentation are excellent. Most of the experiments are explained well, figures are clear and the interpretation appropriate. I think that this manuscript is suitable for publication in Nature

Communications. However, several issues need to be addressed before it is accepted, as listed below:

Major comments
1. The authors explore two very distinct phenotypes – genetic instability of GAA repeats (expansions and contractions) and loss of viability due to the presence of GAA repeats. Overall, I think the data presented suggests that the two phenotypes are not really related to one another. This is partly because there is poor concordance between the two phenotypes in the different double mutants tested. But perhaps the most telling result is the fact that GAGAAGAAA repeats, which do not form triplexes and do not exhibit genetic instability (or at least, do not show contractions in PMID: 31911468. I could not find published data relating to expansions. Do you know if these repeats expand?), still reduce viability in an Mcm10 mutant background. The authors do not sufficiently discuss this result and its important implications. Also, the manuscript is written in a way that makes it seem like these phenotypes are linked. The authors might consider restructuring the manuscript and figures so that they discuss instability and viability as two different, but related, phenotypes.

2. The results of measuring fork rates using the in vivo imaging method are a bit confusing and contradictory. The interpretation also requires some revision. The fact that the mcm10-1 mutant shows reduced replication rate through the no-repeat control makes sense and agrees with the general role of Mcm10 in promoting elongation. However, the results with the repeat tract are a bit surprising and confusing.
First, as the authors point out, they do not observe any difference in fork rates through the GAA tract. I wonder if this relates to another discrepancy seen in this system when looking at replication of G4s (in PMID: 30395308, the authors found that G4s induce a delay only when positioned on the lagging strand template, whereas other evidence suggests G4s induce stalling only when on the leading strand template (e.g. PMID: 21145480, PMID: 21873979, PMID: 37781931). Have you tried the opposite orientation? Are you confident about the assignment of leading/lagging orientation relative to the origin?
Next, the authors find that fork progression through the repeats is slower in the mcm10-1 mutant relative to the WT strain, but not as slow as replication through the no-repeat sequence in the mcm10-1 strain. The authors reach the conclusion that "… the mcm10-1 mutation could lead to faster replication through the repeat-expansion cassette" in the last paragraph of the results section. I'm not sure about this. The result clearly shows that the mutation leads to slower replication through the repeat relative to the WT strain. If anything, the result is that somehow, the repeat tract permits faster fork speeds relative to the no-repeat in the mcm10-1 background, but not in a WT background. In the discussion, the authors provide a better explanation of the result, and here suggest that the presence of Mcm10 slows down replication through the repetitive cassette. However, if this effect is big enough to be picked up in the mcm10-1 background, then in the WT strain one would expect to see slower forks relative to no-repeat with the same magnitude.
So on the one hand, Mcm10 has a general role in speeding up elongation, but on the other hand, it plays a role in slowing down faster-than-normal forks when replicating repeats?
Bottom line, I'm not completely convinced with these results. They don't fully make sense and are confusing, and are also difficult to put together with the rest of the data into a logical model.

Minor comments
1. Page 5, line 113, the first results section mentions that GAA repeats serve as the lagging strand template in humans in the FXN gene. This is not correct. In PMID: 27425605, authors show that when GAA repeats are not expanded, replication forks arrive from both directions. When GAA repeats are expanded, most forks arrive 3' to 5' into the GAA repeats, such that the GAA repeats are the leading strand template.
2. Page 5, line 136. The authors find that the mcm2G44D mutation alone does not affect repeat instability. This is an important finding, as it means that fork slowing per se does not drive repeat instability. It's worth making this point and contrasting it with the Mcm10-1 phenotype, which also leads to fork slowing.
3. In Figure 3, the authors use HU to induce fork uncoupling. However, HU will affect all polymerases, including pol alpha, delta, and epsilon. Therefore, while HU induces uncoupling, it also affects lagging strand synthesis (and any subsequent repair related synthesis). Also, these experiments were carried out at 30° rather than 27°. Why?
4. Page 9, line 227. "…formation of a stable of a triplex…" should read "…formation of a stable triplex…".
5. In Figure 4, the authors have analysed RPA OE for both GAA and GAGAAGAAA repeats, but only GAA repeats for the mcm10-1/mcm2G400D double mutant. Does the mcm2 mutation rescue the reduced viability induced by the GAGAAGAAA repeats?
6. In Figure 5, the authors have analysed various double mutants for expansions. What about contractions?
7. The effect of deleting Rad5 and Exo1 on viability are not convincing at all (I can't see a significant difference on the spot assays in Figure 5A). Perhaps worth carrying out a colony formation assay for these.
8. Page 13, line 327. The authors suggest that the delay in S phase might be due to checkpoint activation. However, it's well established (and they find this too) that Mcm10 loss leads to a reduction in fork rates. So this would obviously lead to slower S phase in general. Perhaps consider western blots of checkpoint effectors?
9. The authors deleted Mrc1. The phenotype might be complicated because Mrc1 plays a role in fork speed as well as checkpoint activation. They might want to consider using the Mrc1AQ mutant (e.g. as they did in PMID: 19136957). Also, have they considered looking at the role of Csm3 and Tof1?
10. Page 16, line 396. "…it could result from the degradation Pol alpha…" should read "degradation of Pol alpha".
11. Page 16, line 409. "…leading strand synthesis by polymerase epsilon synthesis is halted…". The word synthesis occurs twice. The second time should be removed.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The additional experiments further strengthen the conclusions of the manuscript. I especially appreciate that the authors carefully considered of mutant viability, and the validity of their results. I am completely satisfied with the revisions.

Reviewer #2

(Remarks to the Author)
The revised manuscript by Masnovo et al. is much improved over the original submission.
The authors have now included a variety of new experiments and have better structured the text. The in vivo measurements of fork speeds also better integrate with the genetics.

Overall this work is well suited for publication in Nature Comms. I only have various corrections and comments, as well as one key major issue.

The authors uncovered a viability defect in the Mcm10-1 mutant when it harbours long GA-rich repeats in an essential chromosomal location, though the parental Mcm10-1 mutant is already affected and grows slowly. They then generated a variety of double mutants to establish what causes this viability defect. Some double mutants exhibited rescue while others exhibited more severe phenotypes than the respective single mutants. However, it is unclear which of these phenotypes are related to the presence of the GA-rich repeat. For example, combined mutations in pol delta (or loss of Pol32) and Mcm10 could be deleterious regardless of the (GAA)100 repeat because of global replication defects. The same for a combination of Mcm10 mutations and checkpoint mutants. The authors assume that in all double mutants, the phenotypes are linked to the GAA repeat. They can only do that if they also compared viability of single/double mutants that do not harbour a repeat, and not observed the same phenotype. The same holds true for the cell cycle analysis of Mcm10/Mrc1 single/double mutants.

Minor comments:
1. Row 54: "alternative secondary structures" should read "alternative DNA secondary structures"

2. Row 172: "with (GAA)100 serves as" should read "with (GAA)100 serving as". Also - it would be helpful to add GAA and TTC in the schematic in Fig 3A on the respective strands.

3. Row 195: "which lacks" should read "but lacks"

4. Row 197: "than the wild type (GAA)124 strain" should read "than (GAA)124 repeats".

5. Row 200: Another possible explanation is that RPA shows a bias towards certain sequence contexts. Perhaps it binds poorly to GA-rich (or TC-rich) ssDNA.

6. Row 266: Pol32 is not only a processivity factor for pol delta, it is also shared with pol zeta.

7. Row 269: "we observed a synthetic viability defect" should read "we observed synthetic lethality"

8. Row 303: "did not rescue of (GAA)124" should read "did not rescue (GAA)124"

9. Row 320: Did the authors check the expansion/contraction rate in the single Mrc1 deletion? Given the role of Mrc1 in coupling unwinding/synthesis, this single mutant would fit with the section where they deleted Ctf4 or treated with HU.

10. Row 324: "as well as a synthetic viability defect" should read "as well as synthetic lethality"

**Response to reviewers**

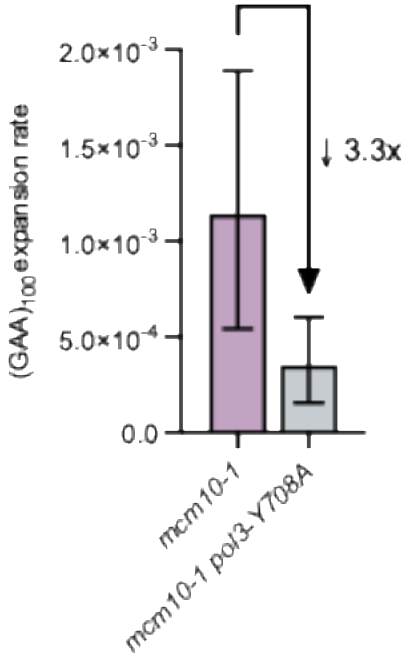
**Reviewer's comments are in black, our responses are in blue**

Reviewer #1 (Remarks to the Author):

The study by Masnovo and coworkers describes an in-depth analysis of the role of Mcm10 in GAA repeat stability in budding yeast. The hypomorphic mcm10-1 mutation causes a more than 30-fold increase in GAA repeat expansions, and a milder 10-fold increase in contractions at the semi-permissive temperature of 27oC. The authors show convincingly that this effect is not due to the degradation of the catalytic subunit of DNA polymerase-alpha. Interestingly, deletion of Ctf4, a binding partner of Mcm10, has no impact on GAA expansion. The effect of mcm10-1 can be reversed by the mcm2-G400D suppressor mutation that alters the Mcm2 subunit of the CMG helicase. This strongly suggests that Mcm10's role in GAA repeat stability has something to do with its function during elongation and is independent of origin firing. Although the authors do not formally exclude that an origin firing defect is not responsible for the phenotype, they show very elegantly that Mcm10 regulates fork speed as the replisome passes through a (GAA)100 repeat cassette. Whereas forks move more slowly through the genome in mcm10-1 mutants, they don't slow down in GAA repeats. Together with the observation that overexpression of RPA in the mcm10-1 mutant background doesn't affect repeat expansion but rescues cell viability, the authors conclude that mcm10-1 accumulates single-stranded DNA and likely leads to RPA exhaustion. This is further corroborated by the finding that both DNA damage tolerance and S phase checkpoint activation promote cell viability at semi-permissive conditions. Exo1 doesn't have a role in expansion, but deletion partially rescues viability, arguing that the gaps generated in mcm10-1 mutants are exonucleolytically processed. All experiments are well controlled and convincing. They fully support the authors claims.

I have a minor comment about the model in Fig. 7C. I agree with the authors that single-stranded DNA likely results from DNA polymerase slippage. This could be directly tested by testing a pol-delta mutant with reduced processivity.

We thank the reviewer for this suggestion and agree that testing the role of DNA Pol δ makes perfect sense. We, thus, tested both the viability and repeat expansion rates in strains with the *pol3-Y708A* mutant and observed a severe synthetic viability defect with the *mcm10-1* mutation in strains with $(GAA)_n$ repeats at all tested temperatures **(Fig. 5d and Supplementary Fig. 5a)**. These data support our original hypothesis that Pol δ performs synthesis of under-replicated DNA in the *mcm10-1* mutant. While measuring the expansion rates in the *mcm10-1 pol3-Y708A* double mutant, we observed a partial rescue in the repeat expansion levels (see below), which also agrees with our hypothesis. However, these double mutant cells were so sick that we cannot consider these results fully reliable. Thus, we did not include them in the revised manuscript.




Prior to the original submission, we had attempted to delete *POL32*, an accessory subunit of Pol δ which increases processivity during both replication and repair, *in mcm10-1* strains, but failed to do so on multiple trials, which hinted to synthetic lethality. To address the reviewer's comment, we therefore decided to replace the endogenous *POL32* promoter with an inducible and tagged *pGAL1* promoter [1]. In accordance with the results above, the *mcm10-1 pGAL1-3xHA-POL32* combination exhibited strong synthetic viability defects when plated on glucose, in which expression of *POL32* is repressed. On the other hand, we did not observe this defect on

galactose, where *POL32* expression is induced **(Fig. 5d)**. We confirmed Pol32 protein expression levels by western blotting with an anti-HA antibody both under repression (glucose) and induction (galactose) conditions **(Supplementary Fig. 5c)**.

There are over 50 human disorders caused by repeat expansions, and this study is the first to demonstrate that Mcm10 modulates replication fork speed through GAA repeats. Mcm10's role in elongation has been highly controversial and this study does not only provide mechanistic insight into GAA repeat expansion but puts a long-standing controversy in the replication field to rest. The results are consistent with other reports demonstrating that human MCM10 controls replication through telomeric repeats and has a direct role in suppressing single-stranded gaps, making it plausible that MCM10 might have a similar role in the control of GAA repeats in human cells.

We appreciate this very enthusiastic comment by the reviewer.

Reviewer #2 (Remarks to the Author):

The manuscript by Masnovo et al. explores the role of the replication factor Mcm10 in the genetic instability of GAA repeats using budding yeast as a model system.
It mainly employs a well-established system from the Mirkin lab for measuring the rate of repeat contractions or expansions, testing various double mutants to try and establish the mechanism by which Mcm10 affects repeat instability. In addition, they authors find that Mcm10 plays an important part in viability of yeast cells when expanded GAA repeats are located on an essential chromosome arm, and also define the genetic requirements for this role. Finally, the authors use live cell imaging to measure fork speeds through expanded GAA repeats in WT vs Mcm10 mutated cells.
The topic and overall experimental design and presentation are excellent. Most of the experiments are explained well, figures are clear and the interpretation appropriate. I think that this manuscript is suitable for publication in Nature Communications. However, several issues need to be addressed before it is accepted, as listed below:

**Major comments**

1. The authors explore two very distinct phenotypes – genetic instability of GAA repeats (expansions and contractions) and loss of viability due to the presence of GAA repeats. Overall, I think the data presented suggests that the two phenotypes are not really related to one another. This is partly because there is poor concordance between the two phenotypes in the different double mutants tested.

**Reply:** We agree with the reviewer that repeat instability and viability phenotypes are not necessarily related in all cases, or at least we cannot exclude a repeat independent effect of the various mutants. We therefore restructured both the data presentation, combining all the viability and growth data into one figure **(Fig. 5)** and the instability data for the double mutants into another figure **(Fig. 6)**, and we revised the text as well.

However, since the viability defects observed in *mcm10-1* are dependent on the length of the repeat as shown in **Fig. 5a and 5b** and **Supplementary Fig. 4**, we do believe that this specific phenotype is directly dependent on the presence and length of a single $(GAA)_n$ repeat at an essential chromosomal location.

But perhaps the most telling result is the fact that GAGAAGAAA repeats, which do not form triplexes and do not exhibit genetic instability (or at least, do not show contractions in PMID: 31911468. I could not find published data relating to expansions. Do you know if these repeats expand?), still reduce viability in an Mcm10 mutant background. The authors do not sufficiently discuss this result and its important implications. Also, the manuscript is written in a way that makes it seem like these phenotypes are linked. The authors might consider restructuring the manuscript and figures so that they discuss instability and viability as two different, but related, phenotypes.

**Reply:** The contraction rate of asymmetrical $(GAGAAGAAA)_n$ repeat is certainly much lower (10-fold) than that of $(GAA)_n$ repeats in the WT strains, as was found both in our previous study [2] and in the current study. This shows that in the WT cells, contractions are strongly promoted by the triplex formation. At the same time, the *mcm10-1* mutation increases contractions of both

repeats in a quantitatively similar manner, which was rescued by RPA overexpression. These data suggest that in the *mcm10-1* mutant, an excess of repetitive ssDNA elevates contraction rates. We have added this data to the revised manuscript (**Supplementary Fig. 1d)**. We also made it clear that both the increase in contractions and the defects in viability are shared between the two repeats.

We have not tested expansions of the $(GAGAAGAAA)_n$ non-mirror repeat to date, as this will be the focus of a separate study in the future.

2. The results of measuring fork rates using the in vivo imaging method are a bit confusing and contradictory. The interpretation also requires some revision. The fact that the mcm10-1 mutant shows reduced replication rate through the no-repeat control makes sense and agrees with the general role of Mcm10 in promoting elongation. However, the results with the repeat tract are a bit surprising and confusing.

First, as the authors point out, they do not observe any difference in fork rates through the GAA tract. I wonder if this relates to another discrepancy seen in this system when looking at replication of G4s (in PMID: 30395308, the authors found that G4s induce a delay only when positioned on the lagging strand template, whereas other evidence suggests G4s induce stalling only when on the leading strand template (e.g. PMID: 21145480, PMID: 21873979, PMID: 37781931). Have you tried the opposite orientation? Are you confident about the assignment of leading/lagging orientation relative to the origin?

**Reply:** All cassettes were sequenced when introduced into the strains, therefore we are confident that the $(GAA)_n$ repeats are on the lagging strand template with regard to *ARS413*. The data analysis for the live-cell microscopy experiments is conducted by measuring the differential in time between the doubling in intensity of the lacO/lacI-Envy (5' to the repeat) and the tetO/tetR-tdTomato (3' to the repeat). Therefore, we only included data in which we know that replication is originating from *ARS413* in the expected direction.

Since we collected all data, including when replication occurs simultaneously at both loci and in which the tetO/tetR-tdTomato replicates before the lacO/lacI-Envy, we created a pie chart analogous to the one found in Dovrat et al., 2018 [3] and added it to the revised manuscript as **Supplementary Fig. 3** (raw data can be found in the Source Data file). This new figure shows

that in the case of the WT strain containing the entire expansion cassette with the $(GAA)_{100}$ repeat, 80% of the forks progress from *ARS413* in a 5'→3' orientation with regards to the cassette. Therefore, in 80% of all cases, the $(GAA)_n$ tract serves as the lagging-strand template.

Next, the authors find that fork progression through the repeats is slower in the mcm10-1 mutant relative to the WT strain, but not as slow as replication through the no-repeat sequence in the mcm10-1 strain. The authors reach the conclusion that "…the mcm10-1 mutation could lead to faster replication through the repeat-expansion cassette" in the last paragraph of the results section. I'm not sure about this. The result clearly shows that the mutation leads to slower replication through the repeat relative to the WT strain. If anything, the result is that somehow, the repeat tract permits faster fork speeds relative to the no-repeat in the mcm10-1 background, but not in a WT background. In the discussion, the authors provide a better explanation of the result, and here suggest that the presence of Mcm10 slows down replication through the repetitive cassette. However, if this effect is big enough to be picked up in the mcm10-1 background, then in the WT strain one would expect to see slower forks relative to no-repeat with the same magnitude.
So on the one hand, Mcm10 has a general role in speeding up elongation, but on the other hand, it plays a role in slowing down faster-than-normal forks when replicating repeats?
Bottom line, I'm not completely convinced with these results. They don't fully make sense and are confusing, and are also difficult to put together with the rest of the data into a logical model.

**Reply:** We are grateful to the reviewer for this insightful comment, which led us to realize that normalizing replication data (bp/sec) for DNA segments of different lengths (one with no expansion cassette and another with the entire expansion cassette) could be misleading, as it assumes that the replication rate is constant for these two segments.
Consequently, we have redone these experiments and are now comparing the raw replication timing in minutes between the two strains carrying either a cassette with the $(GAA)_n$ repeat or the same cassette with a filler sequence, which makes the distance between the two arrays identical, and thus, no normalization is needed. The new results presented in the revised manuscript **(Fig. 3b and Source Data File)** led us to different conclusions that are, in fact, much more in line with the rest of the data presented in the manuscript and our model.

In brief, we now observe repeat-dependent replication slowdown in the wild-type context, consistent with our previously published results. This effect is further exacerbated in the *mcm10-1* mutant. In addition, we observe a slight slowdown in replication in the context of the *mcm10-1* mutation in the no-repeat control strain. Altogether, this indicates that Mcm10 is especially important for replication through the $(GAA)_n$ repeat, diminishing replication stalling. The new results are shown in **Fig. 3** and discussed in the revised Discussion section.

**Minor comments**

1. Page 5, line 113, the first results section mentions that GAA repeats serve as the lagging strand template in humans in the FXN gene. This is not correct. In PMID: 27425605, authors show that when GAA repeats are not expanded, replication forks arrive from both directions. When GAA repeats are expanded, most forks arrive 3' to 5' into the GAA repeats, such that the GAA repeats are the leading strand template.

**Reply:** We thank the reviewer for noticing this, and we apologize for the mistake. We removed the sentence from the revised manuscript. We decided to use this orientation since it is the one that has previously been shown to cause replication fork stalling in both our yeast [1] and human cell model systems [4].

2. Page 5, line 136. The authors find that the mcm2G400D mutation alone does not affect repeat instability. This is an important finding, as it means that fork slowing per se does not drive repeat instability. It's worth making this point and contrasting it with the Mcm10-1 phenotype, which also leads to fork slowing.

**Reply:** We agree with the reviewer that this is an interesting finding on its own. We highlighted this by updating the following sentence in the text in the Results section:

Lines 132-134: "We found that the *mcm2-G400D* mutation alone did not affect repeat instability **(Figs. 2a and 2b)**, indicating that a decrease in the helicase unwinding rate alone is insufficient to trigger repeat instability."

3. In Figure 3, the authors use HU to induce fork uncoupling. However, HU will affect all polymerases, including pol alpha, delta, and epsilon. Therefore, while HU induces uncoupling, it also affects lagging strand synthesis (and any subsequent repair related synthesis). Also, these experiments were carried out at 30° rather than 27°. Why?

**Reply:** We thank the reviewer for this comment and agree that we need to clarify the fact that HU treatment has pleiotropic effects on DNA replication. We added the following to the Discussion section of the revised manuscript:

Lines 369-373: "We acknowledge, however, that hydroxyurea treatment has pleiotropic effects on replication, as it also affects origin firing, checkpoint activation, and the function of Pol δ in lagging strand synthesis. Thus, we cannot confidently attribute the observed effects on repeat instability to fork uncoupling alone."

As for the temperature concern, we conducted these experiments at 30°C because this is the standard temperature at which we run our assays when the treatment/mutant to be tested does not display a temperature sensitive phenotype, and a previous study did not observe temperature sensitivity in *ctf4Δ* cells [5]. In addition, we performed no direct comparison between the experiments in this figure and the ones in Figs. 1 and 2. For these reasons, we believe that the use of 30°C as a temperature for these experiments was appropriate.

4. Page 9, line 227. "…formation of a stable of a triplex…" should read "…formation of a stable triplex…".

**Reply:** We corrected this typo in the revised manuscript (Line 196).

5. In Figure 4, the authors have analysed RPA OE for both GAA and GAGAAGAAA repeats, but only GAA repeats for the mcm10-1/mcm2G400D double mutant. Does the mcm2 mutation rescue the reduced viability induced by the GAGAAGAAA repeats?

**Reply:** We agree that this piece of data was missing to determine whether the *mcm2G400D* mutant can rescue the observed viability phenotypes independently of strong triplex formation. We conducted the suggested experiment and added the result to **Fig. 5b**. We also updated the following sentence to the text:

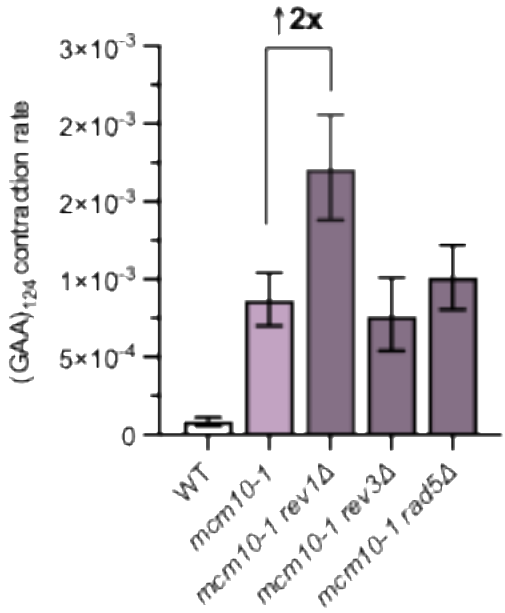
Lines 230-232: "As was observed in the case of repeat instability, the *mcm10-1 mcm2G400D* double mutant fully rescued the viability defects for both types of homopurine-homopyrimidine repeats."

6. In Figure 5, the authors have analysed various double mutants for expansions. What about contractions?

**Reply:** We are happy to provide our reasoning for why we did not proceed with all double mutants in the case of *mcm10-1* contractions: In the original manuscript, we included the *mcm10-1 rad53K227A* mutant for repeat contractions, which has now been moved to the main body as **Fig. 6b** to enhance the contrast. This double mutant did not rescue the elevated contraction rate in the *mcm10-1* mutant, as opposed to what happened in the case of expansions, and therefore excluded a role for the checkpoint in *mcm10-1* mediated contractions.

We also showed that RPA overexpression rescues contractions in the *mcm10-1* mutant, while it does not rescue expansions **(Figs. 2a and 2b)**. These results, combined with the contraction-specific increase in instability in other mutants that promote ssDNA accumulation on the lagging strand, such as *CTF4* deletion (Fig. 3B), suggest that the mechanism of contractions in the case of the *mcm10-1* mutant is consistent with our previously published contraction model [2], and we therefore decided to focus on the mechanism of expansions in the *mcm10-1* background for the rest of the manuscript.

Nevertheless, we have performed some additional contraction experiments with double mutants, which are shown below:




As one can see, there were no differences in the tested double mutants, except for the *mcm10-1 rev1Δ* case. A 4-fold increase in contractions was observed in the *rev1Δ* single mutant in our previous study cited above, and we therefore believe this effect to be independent of Mcm10 and again consistent with our previous model.

7. The effect of deleting Rad5 and Exo1 on viability are not convincing at all (I can't see a significant difference on the spot assays in Figure 5A). Perhaps worth carrying out a colony formation assay for these.

**Reply:** We appreciate the reviewer's concern and agree that these specific double mutants have subtler effects when compared to other ones. We conducted a colony-forming assay as suggested by the reviewer for both the *mcm10-1 exo1Δ* and *mcm10-1 rad5Δ* double mutants **(Supplementary Fig. 5b).** In both cases, we did not observe a difference from the *mcm10-1* single mutant in total number of colonies. Nevertheless, we observed that growth in the *mcm10-1 rad5Δ* was further slowed, while cells from the *mcm10-1 exo1Δ* mutant grew faster than the *mcm10-1* single mutant. We adjusted our claims accordingly and refer to growth rate instead of viability for these mutants in the revised manuscript.

8. Page 13, line 327. The authors suggest that the delay in S phase might be due to checkpoint activation. However, it's well established (and they find this too) that Mcm10 loss leads to a reduction in fork rates. So this would obviously lead to slower S phase in general. Perhaps consider western blots of checkpoint effectors?

**Reply:** We agree with the reviewer that western blots of the Rad53 checkpoint effector are a more direct measure and allow us to directly relate cell-cycle stage with checkpoint status. We detected checkpoint activation in the *mcm10-1* mutant during mid to late S-phase, and it is resolved by G2/M, as determined by correlation with the flow cytometry data **(Figs. 6e and 6f)**. We also conducted western blots with the *mcm10-1 rad9Δ, mcm10-1 mrc1Δ,* and *mcm10-1 mrc1$^{AQ}$* mutants and determined that checkpoint activation was Rad9-mediated, indicating the presence of persistent ssDNA gaps and/or DSBs in the *mcm10-1* mutant.

9. The authors deleted Mrc1. The phenotype might be complicated because Mrc1 plays a role in fork speed as well as checkpoint activation. They might want to consider using the Mrc1AQ mutant (e.g. as they did in PMID: 19136957). Also, have they considered looking at the role of Csm3 and Tof1?

**Reply:** We are exceptionally grateful for the reviewer for this suggestion, as these experiments substantially improved the final quality of our study and our model. In the revised manuscript, we provide expansion rates **(Fig. 6c)** and viability data **(Fig. 6d)** for the combined *mcm10-1 mrc1$^{AQ}$* mutant, as well as its Rad53 phosphorylation state **(Fig. 6e)** and flow cytometry profile **(Fig. 6f)**.

Altogether, we concluded that the phenotypes observed in the *mcm10-1 mrc1Δ* double mutant are dependent on Mrc1's replication-associated function, which changes our model and suggests that the Mrc1 replication function becomes essential for the fork integrity and replication through the repeat in the *mcm10-1* mutant. This agrees with previous studies showing that in the absence of Mrc1, Mcm10 functioning becomes crucial for DNA replication in response to replication stress [6–8]. We now discuss these results in the revised manuscript. We believe that Tof1 and Csm3 would have a similar effect as Mrc1 in this case.

10. Page 16, line 396. "…it could result from the degradation Pol alpha…" should read "degradation of Pol alpha".

**Reply:** We corrected this typo in the revised manuscript (Line 352).

11. Page 16, line 409. "…leading strand synthesis by polymerase epsilon synthesis is halted…". The word synthesis occurs twice. The second time should be removed.

**Reply:** We corrected this typo in the revised manuscript (Line 365).

**References**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2. Khristich, A. N., Armenia, J. F., Matera, R. M., Kolchinski, A. A. & Mirkin, S. M. Large-scale contractions of Friedreich's ataxia GAA repeats in yeast occur during DNA replication due to their triplex-forming ability. *Proc Natl Acad Sci USA* **117**, 1628–1637 (2020).
3. Dovrat, D. *et al.* A Live-Cell Imaging Approach for Measuring DNA Replication Rates. *Cell Reports* **24**, 252–258 (2018).
4. Rastokina, A. *et al.* Large-scale expansions of Friedreich's ataxia GAA•TTC repeats in an experimental human system: role of DNA replication and prevention by LNA-DNA oligonucleotides and PNA oligomers. *Nucleic Acids Research* **51**, 8532–8549 (2023).
5. Ghaddar, N., Luciano, P., Géli, V. & Corda, Y. Chromatin assembly factor-1 preserves genome stability in ctf4Δ cells by promoting sister chromatid cohesion. *Cell Stress* **7**, 69–89 (2023).
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8. McClure, A. & Diffley, J. Rad53 checkpoint kinase regulation of DNA replication fork rate via Mrc1 phosphorylation. *eLife* **10**, e69726 (2021).

**Response to reviewers**

**Reviewer's comments are in black, our responses are in blue**

Reviewer #1 (Remarks to the Author):

The additional experiments further strengthen the conclusions of the manuscript. I especially appreciate that the authors carefully considered of mutant viability, and the validity of their results. I am completely satisfied with the revisions.

**Reply:** We thank the reviewer for the positive feedback and recommendation for publication.

Reviewer #2 (Remarks to the Author):

The revised manuscript by Masnovo et al. is much improved over the original submission. The authors have now included a variety of new experiments and have better structured the text. The in vivo measurements of fork speeds also better integrate with the genetics.

Overall this work is well suited for publication in Nature Comms. I only have various corrections and comments, as well as one key major issue.

The authors uncovered a viability defect in the Mcm10-1 mutant when it harbours long GA-rich repeats in an essential chromosomal location, though the parental Mcm10-1 mutant is already affected and grows slowly. They then generated a variety of double mutants to establish what causes this viability defect. Some double mutants exhibited rescue while others exhibited more severe phenotypes than the respective single mutants. However, it is unclear which of these phenotypes are related to the presence of the GA-rich repeat. For example, combined mutations in pol delta (or loss of Pol32) and Mcm10 could be deleterious regardless of the (GAA)100 repeat because of global replication defects.

**Reply:** We agree with the reviewer that we cannot decisively conclude that viability defects in the double mutants with *mcm10-1* are repeat specific, unlike for the single *mcm10-1* mutant, where we show a repeat length dependent loss of viability. We actually believe that many of the viability defects that are observed in our double mutants are global, rather than repeat specific. That said, while the rescue by *mcm2-G400D* of the *mcm10-1* phenotype at non-permissive temperature was previously demonstrated (PMID: 19917723), we show that it rescues the repeat-specific viability defect as well. Pol32 knockout is deleterious in the *mcm10-1* context (PMID: 11168584), albeit its effect seems to be milder that in our case. It is possible, therefore, that some viability defects in double mutants can be additionally exacerbated by the presence of the repeat, which could be a subject of future investigation. We revised the text in the manuscript in various places to make these points clearer.

The same for a combination of Mcm10 mutations and checkpoint mutants. The authors assume that in all double mutants, the phenotypes are linked to the GAA repeat. They can only do that if they also compared viability of single/double mutants that do not harbour a repeat, and not

observed the same phenotype. The same holds true for the cell cycle analysis of Mcm10/Mrc1 single/double mutants.

**Reply:** In the case of Rad53 mutations, negative interactions with *mcm10-1* have been previously detected (PMID: 11168584). Mrc1 and Rad9 deletion have not been tested before in a no repeat context. We, thus, created an *mcm10-1 mrc1Δ* double mutant in the no repeat control strain and observed a viability defect similar to that observed in the repeat-containing strain, indicating that this effect is not repeat specific. We believe the same would be true for an *mcm10-1 rad9Δ* mutant.

We would like to note, however, that we did not claim any repeat specificity in the case of the flow cytometry data, especially not in the case of the *mcm10-1 mrc1Δ* double mutant. The main reason for this is that the behavior of those strains reflects a classical cell cycle profile of an *mrc1Δ* single mutant, as has been published in strains without the repeats. This is now emphasized in the revised text in lines 351-352. We believe, therefore, that conducting cell-cycle analysis with the *mcm10-1 mrc1Δ* in the no repeat context is not necessary as it would not substantially add to our results.

Minor comments:
1. Row 54: "alternative secondary structures" should read "alternative DNA secondary structures"

**Reply:** We incorporated this suggestion in the revised manuscript.

2. Row 172: "with (GAA)100 serves as" should read "with (GAA)100 serving as". Also - it would be helpful to add GAA and TTC in the schematic in Fig 3A on the respective strands.

**Reply:** We corrected the typo in the revised manuscript, and we have updated Figure 3 to incorporate GAA and CTT on the respective strands as suggested.

3. Row 195: "which lacks" should read "but lacks"

**Reply:** We corrected this in the revised manuscript.

4. Row 197: "than the wild type (GAA)124 strain" should read "than (GAA)124 repeats".

**Reply:** We corrected this in the revised manuscript.

5. Row 200: Another possible explanation is that RPA shows a bias towards certain sequence contexts. Perhaps it binds poorly to GA-rich (or TC-rich) ssDNA.

**Reply:** It is indeed the case that RPA has a 10-fold lower affinity for poly-purine DNA sequences than it does for poly-pyrimidine DNA sequences (PMID: 11278662), and the poly-purine GAA and GAGAAGAAA sequences are on the lagging strand template, which could exacerbate the effect on repeat contractions. We discuss this in the revised manuscript (Lines 404-406).

6. Row 266: Pol32 is not only a processivity factor for pol delta, it is also shared with pol zeta.

**Reply:** Thank you for pointing this out. We have added this information to the revised manuscript (Lines 274-275) and added the appropriate citation.

7. Row 269: "we observed a synthetic viability defect" should read "we observed synthetic lethality"

**Reply:** We corrected this in the revised manuscript.

8. Row 303: "did not rescue of (GAA)124" should read "did not rescue (GAA)124"

**Reply:** We corrected this typo in the revised manuscript.

9. Row 320: Did the authors check the expansion/contraction rate in the single Mrc1 deletion? Given the role of Mrc1 in coupling unwinding/synthesis, this single mutant would fit with the section where they deleted Ctf4 or treated with HU.

**Reply:** We agree that this is a good addition and would allow us to compare the effect of Mrc1 deletion on repeat instability in a wild-type context versus an *mcm10-1* background. We have conducted the repeat instability assays with the single *mrc1Δ* mutant that showed a small, statistically insignificant increase in the expansion rate and a significant increase in the contraction rate, comparable to that observed in the Ctf4 knockout (these data are added to Fig. 4 and discussed in the corresponding results section of the revised manuscript). Therefore, we conclude that the replication function of Mrc1 prevents repeat instability in the WT cells similarly to other proteins with a coupling function. At the same time in the *mcm10-1* context, the Mrc1 replication function promotes repeat expansions.

10. Row 324: "as well as a synthetic viability defect" should read "as well as synthetic lethality"

**Reply:** We corrected this typo in the revised manuscript.