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Rtt105 functions as a chaperone for Replication Protein A to preserve genome stability

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Editor correspondence: 23rd February 2018

Thank you for submitting your manuscript on Rtt105 and RPA chaperoning to The EMBO Journal. We have now received reports from three expert referees, which I am enclosing below for your information. As you will see, all referees consider your results potentially interesting, but referees 2 and 3 also raise some significant concerns that I feel would need to be addressed in order to make the study a strong candidate for an EMBO Journal article. In particular, there are concerns that the specific molecular function of Rtt105 that determines its phenotype has not been decisively clarified. Moreover, the referees are worried about the subtle functional phenotypes observed in cells lacking Rtt105, and I am myself wondering how this may be reconciled with the phenotypes ("inviable") reported for Rtt105 deletion in the yeast genome database?

Since it it not clear whether and how these issues could be satisfactorily clarified during a regular single-round revision, I would appreciate hearing from you how you might address/respond to the referees' points should you be given the opportunity to revise this work for The EMBO Journal. Therefore, please carefully consider the attached reports and send back a brief point-by-point response outlining how the referees' comments might be addressed/clarified. These tentative response (parts of which we may choose to share and discuss with referees) would be taken into account when making our final decision on this manuscript. It would be great if you could get back to me with such a response over the course of the coming week.

REFEREE REPORTS

Referee #1 (Report for Author)

This study by Li et al. addresses the role of the S. cerevisiae Rtt105 protein. They find that Rtt105 physically associates with RPA, a ssDNA binding heterotrimeric complex, and with Kap95, the importin beta subunit that functions in nuclear protein import. They show that Rtt105 directly interacts with RPA and is required for RPA nuclear localization, presumably via mediation of RPA-Kap95 interaction. Since RPA is essential for replication, they find, accordingly, that absence of Rtt105 leads to replication stress sensitivity. Interestingly, they find that when RPA is modified with a nuclear localization signal (bypassing the Kap95 importin system), RPA recruitment at replication forks remains defective, indicating that Rtt105 plays an extra role. Through in vitro ensemble and single-molecule assays they find that Rtt105 promotes/facilitates loading of RPA on ssDNA and detaches from RPA once the latter is bound to ssDNA. They find that treatment of rtt105delta cells with agents that induce

replication stress is toxic and leads to genome alterations. They conclude that Rtt105 is important for maintaining genome integrity under these conditions. Overall, this is a well-documented and controlled study and the conclusions are convincing.

- minor concerns

To fully support the model (Figure 7), could the interaction of Rtt115 with Kap95 in the cytoplasmic fraction but not in the nuclear fraction be confirmed?

We noticed that Rtt105 associates with Rim1 (Table EV1). Since Rim1 is a ssDNA binding protein important for mitochondrial DNA replication, could Rtt105 also be important for mitochondrial genome integrity maintenance?

Referee #2 (Report for Author)

Through an exhaustive study that involves a large number of in vivo and in vitro approaches, Li and colleagues report in this work the function of yeast Rtt105; their findings are novel and sound: Rtt105 physically interacts with RPA, escorts it to the nucleus (in part by facilitating the interaction between RPA and its importin) and promotes the loading and mode of binding of RPA at replication forks. According to these functions the absence of Rtt105 causes genetic instability and makes cells sensitive to DNA damaging agents. From these results, the authors conclude that Rtt105 is a chaperone for RPA that is important for genetic stability. The manuscript is well written, concise, and with clear objectives; the experiments have been carefully performed and lead to important conclusions to understand the biology of RPA, an essential and key complex in DNA replication, checkpoint activation and DNA repair. However, some questions remain unclear that should be addressed before publication.

Major concerns

1. My major concern is the apparent contradiction between the levels of RPA at replicating forks and the rate of DNA synthesis (and cell fitness) under unperturbed conditions. Authors claim in discussion that cells are likely to tolerate low levels of RPA in most chromatin regions, and support this conclusion with the work by Toledo et al. However, these authors reduce the levels of RPA to approximately the half, thereby sensitizing only to HU, which exposes more ssDNA and demands more protection. By contrast, the amount of RPA at both the nucleus (by fluorescence) and chromatin (by ChIP) in rtt105 is barely detectable. If the authors are right they should be able to reduce Rfa1 to similar levels as those shown by rtt105 without affecting viability. Alternatively, the length of ssDNA at forks in rtt105 might be shorter than in the wt, or another protein might protect the forks under conditions of RPA limitation. A candidate is Rad51, which competes with RPA for binding to ssDNA and travels with the fork under unperturbed conditions (González-Prieto et al. 2013; EMBO J).

In addition, a parallel ChIP against another component of the replisome would be a nice control of replisome integrity under unperturbed conditions.

2. The authors show convincing in vitro results that Rtt105 does not remain at the ssDNA after loading RPA. However, a ChIP assay is missing showing that Rtt105 is not detected in vivo either.

3. In discussion the authors attribute the sensitivity to DNA damaging agents and the accumulation of spontaneous DNA damage to the role of Rtt105 in DNA replication. They should consider the possibility that the sensitivity to DNA damage agents - and part of the accumulation of spontaneous lesions - might be due to a role of Rtt105 in loading RPA at the ssDNA fragments generated upon processing of DNA lesions (a replication-independent function). In this regard, it would further extend the implications of Rtt105 in genetic stability if the authors analyzed RPA accumulation at a HO-induced DSB by ChIP analysis. Indeed, and connected to point 1, how is the checkpoint response to induced DNA damage (e.g., MMS)? RPA is required for Mec1/Ddc2 recruitment and Rad53 activation and therefore a reduction in RPA loading should, in principle, affects this response.

Minor points

- Figures 2 and 6. Include the profile of RPA and BrdU of a whole chromosome (as supplementary information)
- Figure 3. Panels B and C are changed (correct also in the legend and the main text)
- Figure 4D. Relocate Rfa3 marker
- Figure 5C, 5D and 5E: Are statistically significant the differences?
- Figure 5D: are N=143 and 80 right? (Instead of 99 and 65)
- Legend Fig. EV4 (C): Rtt105 increased the RPA-mCherry binding to ssDNA (?) According to the panel and the main text Rtt105 does not affect this parameter
- Legend EV5 (B) refers to Fig 6G (it is 6H)
- Page 7 (Appendix Fig S2B should be S2C)
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- Page 9: the length of ssDNA in the second chase increased ~2.5 and ~4.5 according to Figure 5E (no 1.5 and 5)
- Page 10: Fig EV3A should be Fig S1A

Referee #3 (Report for Author)

In this manuscript, the authors show that Rtt105 interacts with and regulates RPA in the budding yeast. Their

results convincingly show that Rtt105 interacts with the RPA complex. In rtt105 null cells, the binding of RPA to replication forks is reduced, and genomic instability was increased. Rtt105 is required for the efficient nuclear import of RPA. Rtt105 also stimulates the binding of RPA to ssDNA in vitro. In the DNA curtain assay, Rtt105 enhances the stretching of RPA-bound ssDNA. The authors propose an interesting model in which Rtt105 functions as a chaperon of RPA to facilitate its function in the replication stress response. Although many results in study are quite interesting, the model still needs to be substantiated by additional evidence. Several important questions about the model have to be satisfactorily addressed.

Specific comments:
1. The possibility that the reduction in RPA at replication forks in rtt105 cells is caused by defective replication is not convincingly ruled out.

In 2B and 2C, RPA binding to replication forks was reduced in HU treated rtt105 null cells in early S phase. However, in 6A and 6B, BrdU was also reduced at forks under the same condition. It is impossible to exclude the possibility that the defective binding of RPA to forks is due to initiation problems.

In 2D and 2E, RPA binding to forks was clearly reduced in rtt105 cells at 16C. However, in 6C and 6D, BrdU incorporation at forks was not defective. Do these results suggest that the role of Rtt105 in enhancing RPA binding is not required for replication? If the role of Rtt105 is specific to the response to genomic instability, the assays for genomic instability should be done at 16C because RPA binding is clearly defective under this condition (2D and 2E). The assays in 6E-G were done at 25C.

The experiments done at 25C are somewhat confusing. In 2F, the binding of RPA to forks was clearly reduced in rtt105 cells. However, cell cycle progression was not affected under this condition (S1A). In 6E and 6F, DNA damage accumulated in these cells and the checkpoint was activated. Do these results suggest that the reduction of RPA binding in rtt105 cells does not matter for replication? Is the genomic instability and checkpoint activation in rtt105 cells too weak to slow down S phase? If so, the functional phenotypes of rtt105 cells are quite weak - this is a problem for the overall impact of the paper.

2. The results in Fig. 3 clearly show that the nuclear import of RPA is impaired in rtt105 cells. However, there is no data showing that this specific function of Rtt105 is important for the function of RPA. It is necessary to separate this function of Rtt105 and the other functions in RPA-ssDNA regulation .

3. Several interesting observations were made in the in vitro ssDNA binding EMSAs and DNA curtain assays. However, it is unclear whether and how these observations can be related to each other. In the ssDNA binding assay, only one short ssDNA oligomer (30 nt) was tested. It is not clear whether the effects of Rtt105 on RPA are dependent on the length of ssDNA. In the DNA curtain assay, the binding of RPA-mCherry to ssDNA did not appear to be stimulated by Rtt105, which is not quite consistent with the EMSA results. If the stretching of RPA-ssDNA is distinct from the enhanced RPA binding to ssDNA, how can these functions be separated? The Rtt105 EL is defective for multiple functions and cannot distinguish these possibilities. After all, we still don't know which of the functions of Rtt105 is important for the phenotypes of rtt105 null cells.

We really appreciate all the referees' constructive comments on our manuscript. Below are our point-by-point responses to each referee's comments:

Referee #1 (Report for Author)

This study by Li et al. addresses the role of the S. cerevisiae Rtt105 protein. They find that Rtt105 physically associates with RPA, a ssDNA binding heterotrimeric complex, and with Kap95, the importin beta subunit that functions in nuclear protein import. They show that Rtt105 directly interacts with RPA and is required for RPA nuclear localization, presumably via mediation of RPA-Kap95 interaction. Since RPA is essential for replication, they find, accordingly, that absence of Rtt105 leads to replication stress sensitivity. Interestingly, they find that when RPA is modified with a nuclear localization signal (bypassing the Kap95 importin system), RPA recruitment at replication forks remains defective, indicating that Rtt105 plays an extra role. Through in vitro ensemble and single-molecule assays they find that Rtt105 promotes/facilitates loading of RPA on ssDNA and detaches from RPA once the latter is bound to ssDNA. They find that treatment of rtt105delta cells with agents that induce replication stress is toxic and leads to genome alterations. They conclude that Rtt105 is important for maintaining genome integrity under these conditions. Overall, this is a well-documented and controlled study and the conclusions are convincing.

Response: We thank the referee for his/her time and efforts for reviewing our manuscript and highly appreciate the very positive comments on this exciting story.

- minor concerns

To fully support the model (Figure 7), could the interaction of Rtt115 with Kap95 in the cytoplasmic fraction but not in the nuclear fraction be confirmed?

Response: We thank for the referee to point this out. Based on the SGD database, Kap95 is localized in cytoplasm and nuclear periphery. After thinking about the referee's comment, we realize that it may not appropriate to draw free Kap95 in the nucleus. Therefore, we will modify the cartoon and delete the Kap95 in the nuclear. Moreover, we plan to test where the interaction of Kap95 with Rtt105 occurs using split GFP based bimolecular fluorescence complementation (BiFC) analysis in living cell**.**

We noticed that Rtt105 associates with Rim1 (Table EV1). Since Rim1 is a ssDNA binding protein important for mitochondrial DNA replication, could Rtt105 also be important for mitochondrial genome integrity maintenance?

Response: We thank for the referee to point this out. We will discuss this interesting idea in the revised text.

Referee #2 (Report for Author)

Through an exhaustive study that involves a large number of *in vivo* and *in vitro* approaches, Li and colleagues report in this work the function of yeast Rtt105; their findings are novel and sound: Rtt105 physically interacts with RPA, escorts it to the nucleus (in part by facilitating the interaction between RPA and its importin) and promotes the loading and mode of binding of RPA at replication forks. According to these functions the absence of Rtt105 causes genetic instability and makes cells sensitive to DNA damaging agents. From these results, the authors conclude that Rtt105 is a chaperone for RPA that is important for genetic stability. The manuscript is well written, concise, and with clear objectives; the experiments have been carefully performed and lead to important conclusions to understand the biology of RPA, an essential and key complex in DNA replication, checkpoint activation and DNA repair. However, some questions remain unclear that should be addressed before publication.

Response: We thank the referee for his/her time and efforts for reviewing our manuscript and highly appreciate for the very positive and constructive comments on this exciting story. We have additional data and will also perform new experiments to address the referee's concerns.

Major concerns

1. My major concern is the apparent contradiction between the levels of RPA at replicating forks and the rate of DNA synthesis (and cell fitness) under unperturbed conditions. Authors claim in discussion that cells are likely to tolerate low levels of RPA in most chromatin regions, and support this conclusion with the work by Toledo et al. However, these authors reduce the levels of RPA to approximately the half, thereby sensitizing only to HU, which exposes more ssDNA and demands more protection. By contrast, the amount of RPA at both the nucleus (by fluorescence) and chromatin (by ChIP) in rtt105 is barely detectable. If the authors are right they should be able to reduce Rfa1 to similar levels as those shown by rtt105 without affecting viability. Alternatively, the length of ssDNA at forks in rtt105 might be shorter than in the wt, or another protein might protect the forks under conditions of RPA limitation. A candidate is Rad51, which competes with RPA for binding to ssDNA and travels with the fork under unperturbed conditions (González-Prieto et al. 2013; EMBO J).

Response: We thank the referee's comments and understand the referee's concerns. I would like to point out the following facts for the referee to consider.

First, we do not know the amount of RPA associated with DNA replication forks in *rtt105*Δ mutant cells under unperturbed conditions even if we observed a dramatic reduction of RPA binding at replication forks in *rtt105*Δ cells under this condition (Fig

2D-F). Moreover, it is not very clear on the basal RPA molecules that are needed for normal DNA replication in yeast cells.

Second, it is possible that yeast cells and mammalian cells could tolerate reduced levels of RPA differently. It was established that yeast cells have a higher tolerance to the dNTP level changes comparing with the mammalian cells. Therefore, we agree with the referee that it may not be a fair comparison of the RPA levels in *rtt105*Δ cells and the partial reduction of RPA in mammalian cells in Toledo et al' work. To address this concern, we will modify the text accordingly.

Third, we show that *rtt105*Δ mutant cells process slowly through S phase (5-10 min slower than wild type yeast cells (Appendix Fig S1A)). While this defect is mild, we would like to point out that several proteins involved in DNA replication including Mrc1 (Osborn & Elledge, 2003), Ctf4 (Tanaka et al, 2009; Wang et al, 2010) and Rrm3 (Syed et al, 2016) exhibit mild delay in S phase when mutated and yet are very important for DNA replication.

Fourth, a genome-wide study (Collins et al, 2007) show that the *rtt105*Δ mutation is synthetic lethal with mutations in genes relevant to DNA replication. We also observed *rtt105*Δ mutant exhibits synthetic defects with mutations at genes involved in DNA replication including Orc2, Pol delta and Tof1 (**Letter Fig 2**). If you think that the results are necessary, we could include all the data in the Extended Figures.

Therefore, while it is well established that RPA (literally "*Replication* Protein A") is essential for DNA replication in eukaryotes (Fairman & Stillman, 1988; Wobbe et al, 1987; Wold & Kelly, 1988; Yeeles et al, 2015), we think that Rtt105 does not have an *essential* role in DNA replication. There are two non-exclusive explanations for the small phenotype of *rtt105*Δ mutant cells. 1) It is possible that another RPA chaperone delivers RPA under normal growth conditions. This is not a far-fetch possibility. For instance, histone chaperone CAF-1 is critical for DNA replication-coupled nucleosome assembly from yeast to human cells, and yet yeast cells lacking CAF-1 show very minor growth phenotypes because of compensations from two other histone chaperones, Rtt106 and FACT (Li et al, 2008; Yang et al, 2016). 2) As proposed in our discussion, Rtt105 may be important to promote RPA binding during DNA replication stress. Supporting this idea, we found that replication defects in *rtt105*Δ as measured by BrdU incorporation are much more severe under HU treated condition than normal condition. We will discuss all these points in the revised text.

To test referee's concern further, we will perform the Rad51 ChIP as suggested by the referee.

In addition, a parallel ChIP against another component of the replisome would be a nice control of replisome integrity under unperturbed conditions.

Response: We appreciate the referee's suggestions. In fact, we analyzed how Mcm6 and Cdc45, two components of active replicative helicase, CMG complex, under normal conditions **(Letter Fig 3).** We observed that the association of Mcm6 and Cdc45 with DNA replication forks was not compromised in *rtt105*Δ mutant cells apparently, further supporting our idea that deletion of *RTT105* affects RPA association with DNA replication forks directly. We will include this data into the revised manuscript.

2. The authors show convincing in vitro results that Rtt105 does not remain at the ssDNA after loading RPA. However, a ChIP assay is missing showing that Rtt105 is not detected in vivo either.

Response: We thank the referee's suggestions. We have attempted to perform Rtt105-ChIP. However, we could not detect Rtt105 at DNA replication origins despite repeated attempts. While this result supports the above idea, we did not include this "negative" result as we do not have a positive control site for Rtt105 ChIP.

3. In discussion the authors attribute the sensitivity to DNA damaging agents and the accumulation of spontaneous DNA damage to the role of Rtt105 in DNA replication. They should consider the possibility that the sensitivity to DNA damage agents - and part of the accumulation of spontaneous lesions - might be due to a role of Rtt105 in loading RPA at the ssDNA fragments generated upon processing of DNA lesions (a replication-independent function). In this regard, it would further extend the implications of Rtt105 in genetic stability if the authors analyzed RPA accumulation at a HO-induced DSB by ChIP analysis. Indeed, and connected to point 1, how is the checkpoint response to induced DNA damage (e.g., MMS)? RPA is required for Mec1/Ddc2 recruitment and Rad53 activation and therefore a reduction in RPA loading should, in principle, affects this response.

Response: We appreciate the referee's suggestions and insightful thoughts. We agree with the referee that we cannot exclude the possibility that DNA damage sensitivity of *rtt105*Δ mutant cells is due to processing of DNA lesions and/or DNA damage response. In fact, we have some preliminary results showing that *rtt105*Δ cells was sensitive to HO induced DSBs **(Letter Fig 4A)**. Moreover, both *rad52*Δ *rtt105*Δ and *yku80*Δ *rtt105*Δ double mutant cells showed a synthetic growth defect **(Letter Fig 4B)**. Hence, Rtt105 may not dedicate solely to the DNA replication process. While we agree with the referee that it would be very interesting to determine whether Rtt105 has a role in DNA processing and DNA damage response, we felt that these aspects of studies are beyond the scope of the current study. Thus, we will follow referee's suggestion and discuss the potential roles of Rtt105 in processes mentioned above by the referee in the discussion.

Minor points

- Figures 2 and 6. Include the profile of RPA and BrdU of a whole chromosome (as supplementary information)
Response: We appreciate for this suggestion and we will include these results.

- Figure 3. Panels B and C are changed (correct also in the legend and the main text)
Response: We are sorry for this oversight. We will change it.

- Figure 4D. Relocate Rfa3 marker
Response: We are sorry for this oversight. We will correct it.

- Figure 5C, 5D and 5E: Are statistically significant the differences?
Response: Yes, they are. We will add the p-value in the revised text.

- Figure 5D: are N=143 and 80 right? (Instead of 99 and 65)
Response: We are sorry that we didn't describe this clear. During the analysis of the ssDNA stretching events, some of the ssDNA molecules exhibit two stretching events (**Letter Fig 5**). Thus, the total number of stretching events is higher than the number of ssDNA molecules. We will describe in more details in the methods.

- Legend Fig. EV4 (C): Rtt105 increased the RPA-mCherry binding to ssDNA (?) According to the panel and the main text Rtt105 does not affect this parameter
Response: We are sorry for this oversight and we will modify this.

- Legend EV5 (B) refers to Fig 6G (it is 6H)
Response: We are sorry for this oversight. We will correct it.

- Page 7 (Appendix Fig S2B should be S2C)
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Response: We are sorry for this oversight. We will correct it.

- Page 9: the length of ssDNA in the second chase increased ~2.5 and ~4.5 according to Figure 5E (no 1.5 and 5)
Response: we will clarify the language.

- Page 10: Fig EV3A should be Fig S1A
Response: We are sorry for this oversight. We will correct it.

Referee #3 (Report for Author)

In this manuscript, the authors show that Rtt105 interacts with and regulates RPA in the budding yeast. Their results convincingly show that Rtt105 interacts with the RPA complex. In rtt105 null cells, the binding of RPA to replication forks is reduced, and genomic instability was increased. Rtt105 is required for the efficient nuclear import of RPA. Rtt105 also stimulates the binding of RPA to ssDNA in vitro. In the DNA curtain

assay, Rtt105 enhances the stretching of RPA-bound ssDNA. The authors propose an interesting model in which Rtt105 functions as a chaperon of RPA to facilitate its function in the replication stress response. Although many results in study are quite interesting, the model still needs to be substantiated by additional evidence. Several important questions about the model have to be satisfactorily addressed.

Response: We thank the referee's time and efforts to review our manuscript and highly appreciate for the very encouraging comments on this exciting story.

Specific comments:
1. The possibility that the reduction in RPA at replication forks in rtt105 cells is caused by defective replication is not convincingly ruled out.

In 2B and 2C, RPA binding to replication forks was reduced in HU treated rtt105 null cells in early S phase. However, in 6A and 6B, BrdU was also reduced at forks under the same condition. It is impossible to exclude the possibility that the defective binding of RPA to forks is due to initiation problems.

Response: We appreciate the referee's suggestion. As the referee stated below and as shown in our original manuscript, we have shown that under normal growth conditions without HU, DNA synthesis as measured by BrdU IP-seq was not affected to a detectable degree in *rtt105*Δ mutant cells, whereas the association of RPA with DNA replication forks was reduced. In the presence of HU, we observed that both the association of RPA with DNA replication forks and DNA synthesis were reduced in *rtt105*Δ mutant cells. I agree with the referee that we couldn't rule out that the reduction of RPA with DNA replication forks likely arose also from the reduced DNA synthesis. To address this concern, we analyzed the binding of Mcm6 and Cdc45, two components of active replicative helicase, CMG complex, at replication forks under normal conditions. We observed that the association of Cdc45 and Mcm6 with DNA replication origins at early S phase was not affected apparently under normal growth conditions **(Letter Fig 3)**. Therefore, combining with our *in vitro* data, we think that the reduction of RPA association with DNA replication forks in *rtt105*Δ mutant cells is unlikely due to impaired DNA initiation.

In 2D and 2E, RPA binding to forks was clearly reduced in rtt105 cells at 16C. However, in 6C and 6D, BrdU incorporation at forks was not defective. Do these results suggest that the role of Rtt105 in enhancing RPA binding is not required for replication? If the role of Rtt105 is specific to the response to genomic instability, the assays for genomic instability should be done at 16C because RPA binding is clearly defective under this condition (2D and 2E). The assays in 6E-G were done at 25C.

Response: We thank referee's suggestions. First, we would like to point out that it is standard practice in the DNA replication field to use 16ºC to slow down DNA synthesis in order to monitor the association of DNA proteins with DNA replication forks genome-

wide without using HU (Aparicio et al, 1997). According to previous reports, while DNA replication forks progress slowly on average than their maximal rate, late origins fire and cells proceed through the cell cycle, suggesting that cells do not experience a detectable DNA replication stress at 16ºC (Aparicio et al, 1997; Yu et al, 2014). Therefore, we adopted this condition for our ChIP-seq analysis. Furthermore, we also performed Rfa1 ChIP-qPCR to confirm the reduced RPA binding at replication forks at unperturbed condition at 25ºC (Fig 2F). Therefore, we do not think that it is a concern to analyze RPA binding and DNA synthesis at 16ºC.

To address this concern further, we will perform dot assays and monitor cell growth at 16ºC.

The experiments done at 25C are somewhat confusing. In 2F, the binding of RPA to forks was clearly reduced in rtt105 cells. However, cell cycle progression was not affected under this condition (S1A). In 6E and 6F, DNA damage accumulated in these cells and the checkpoint was activated. Do these results suggest that the reduction of RPA binding in rtt105 cells does not matter for replication? Is the genomic instability and checkpoint activation in rtt105 cells too weak to slow down S phase? If so, the functional phenotypes of rtt105 cells are quite weak - this is a problem for the overall impact of the paper.

Response: We appreciate and understand the reviewer's concern. While the effect of *rtt105*Δ on the DNA synthesis at a global level is small under normal growth conditions compared to under conditions with HU treatment, we present multiple lines of evidence supporting the idea that Rtt105 does have a role in DNA replication.

First, we show that *rtt105*Δ mutant cells progress slowly through S phase (5-10 min slower than wild type yeast cells, Appendix Fig S1A). While this defect is mild, we would like to point out that many proteins involved in DNA replication including Mrc1 (Osborn & Elledge, 2003), Ctf4 (Tanaka et al, 2009; Wang et al, 2010) and Rrm3 (Syed et al, 2016) exhibit mild delay in S phase when mutated and yet are very important to DNA replication.

Second, a genome-wide study (Collins et al, 2007) show that the *rtt105*Δ mutation is synthetic lethal with mutations in genes relevant to DNA replication. We also observed *rtt105*Δ mutant exhibit synthetic defects with mutations at genes involved in DNA replication (**Letter Fig 2**). We will include this result if the referee thought that it is necessary.

Therefore, while RPA (literally "*Replication* Protein A") is essential for DNA replication in eukaryotes (Fairman & Stillman, 1988; Wobbe et al, 1987; Wold & Kelly, 1988; Yeeles et al, 2015), we think that Rtt105 does not have an *essential* role in DNA replication. We would like to point out that the mild phenotype of *rtt105*Δ mutant cells under normal growth conditions is likely due to the following two non-exclusive possibilities. First, it is possible that another RPA chaperone delivers RPA under

normal growth conditions. This is not a far-fetch possibility. For instance, histone chaperone CAF-1 is critical for nucleosome assembly of new H3-H4 from yeast to human cells, and yet yeast cells lacking CAF-1 show very minor growth phenotypes because of compensations from two other histone chaperones, Rtt106 and FACT (Li et al, 2008; Yang et al, 2016). Second, Rtt105 may be play a more important role in DNA replication under replication stress. Supporting this idea, we found that that the replication defects in *rtt105*Δ as measured by BrdU incorporation are much more severe under HU. Therefore, we propose that Rtt105 may be important to regulate RPA under replication stress in the original manuscript.

As we know, DNA replication stress can be caused by a variety of internal agents and external agents as well as chromatin structures. In human cells, oncogene activation can also lead to DNA replication stress. Therefore, cells have devoted many genes to deal with replication stress, and it is very important for cells to deal with replication stress. Our results indicate that Rtt105 is important to regulate the function of RPA during DNA replication stress. In revised manuscript, we will discuss these points and emphasize the role of Rtt105 during replication stress.

2. The results in Fig. 3 clearly show that the nuclear import of RPA is impaired in rtt105 cells. However, there is no data showing that this specific function of Rtt105 is important for the function of RPA. It is necessary to separate this function of Rtt105 and the other functions in RPA-ssDNA regulation.

Response: It was reported that Kap95 is required for nuclear import of RPA. We also found that Rtt105 is important for the Kap95-RPA interaction. We have attempted to map Rtt105 binding site for Kap95 and for RPA. Unfortunately, we have not been able to achieve such a goal, which is not surprising in hindsight because it is likely that Kap95-RPA-Rtt105 form a trimer and the complex formation interaction depends on each other. We agree with the reviewer that it is interesting to determine the RPA nuclear import function of Rtt105 on RPA binding to ssDNA. As RPA binding to ssDNA at DNA replication forks occurs in the nucleus, it is expected that a defect in nuclear import will affect DNA binding of RPA. To test this idea using another means, we decided to fuse a NLS signal on Rfa1 to enable RPA enter the nucleus and thereby bypass the nuclear import function of Rtt105. Surprisingly, we observed that Rtt105 has a role in loading RPA to DNA replication forks. Because this novel function has not been observed in other known RPA binding proteins, we decided to focus on our studies on this unexpected function of Rtt105 in the manuscript. At the same time, we conclude that Rtt105 contributes to both RPA nuclear import and RPA loading at ssDNA substrates.

In the revised manuscript, we will make these points clear in the discussion as well as in the result section.

3. Several interesting observations were made in the in vitro ssDNA binding EMSAs

and DNA curtain assays. However, it is unclear whether and how these observations can be related to each other. In the ssDNA binding assay, only one short ssDNA oligomer (30 nt) was tested. It is not clear whether the effects of Rtt105 on RPA are dependent on the length of ssDNA. In the DNA curtain assay, the binding of RPA-mCherry to ssDNA did not appear to be stimulated by Rtt105, which is not quite consistent with the EMSA results. If the stretching of RPA-ssDNA is distinct from the enhanced RPA binding to ssDNA, how can these functions be separated? The Rtt105 EL is defective for multiple functions and cannot distinguish these possibilities. After all, we still don't know which of the functions of Rtt105 is important for the phenotypes of rtt105 null cells.

Response: We thank the referee's several suggestions and comments. First, we will perform EMSA assay using different length of ssDNA substrates and determine whether ssDNA length affects Rtt105's ability to promote RPA binding to ssDNA. Second, we would like to point out that we might have misled the referee to believe that the *in vitro* EMSA assay result and ssDNA curtain result represent different function of Rtt105 in the original manuscript. Based on the EMSA analysis, more RPA was retained on ssDNA in the presence of Rtt105 than in the absence of Rtt105. This effect would be due to either increased binding affinity of RPA to ssDNA or altered conformational changes in the presence of Rtt105. Our ssDNA curtain assays support the later possibility. Therefore, we suggest that Rtt105 could alter the conformation of RPA so that RPA can efficiently bind to ssDNA *in vitro*. This conclusion is also supported by our *in vivo* results showing that the association of RPA with ssDNA at DNA replication forks is reduced. In the revised manuscript, we will make these points clear.

I agree with the referee that it remains possible that the increased stretch rate of RPA and enhanced binding to ssDNA in EMSA assays could be due to distinct impact of Rtt105 on RPA. Nonetheless, our *in vitro* results show that all these effects depend on the Rtt105-RPA interaction because Rtt105 EL mutant that cannot bind RPA *in vitro* does not exhibit any of these effects on RPA. Taken together, these results strongly support our conclusion that in addition to mediating nuclear import of RPA, Rtt105 has a role in loading RPA onto ssDNA.

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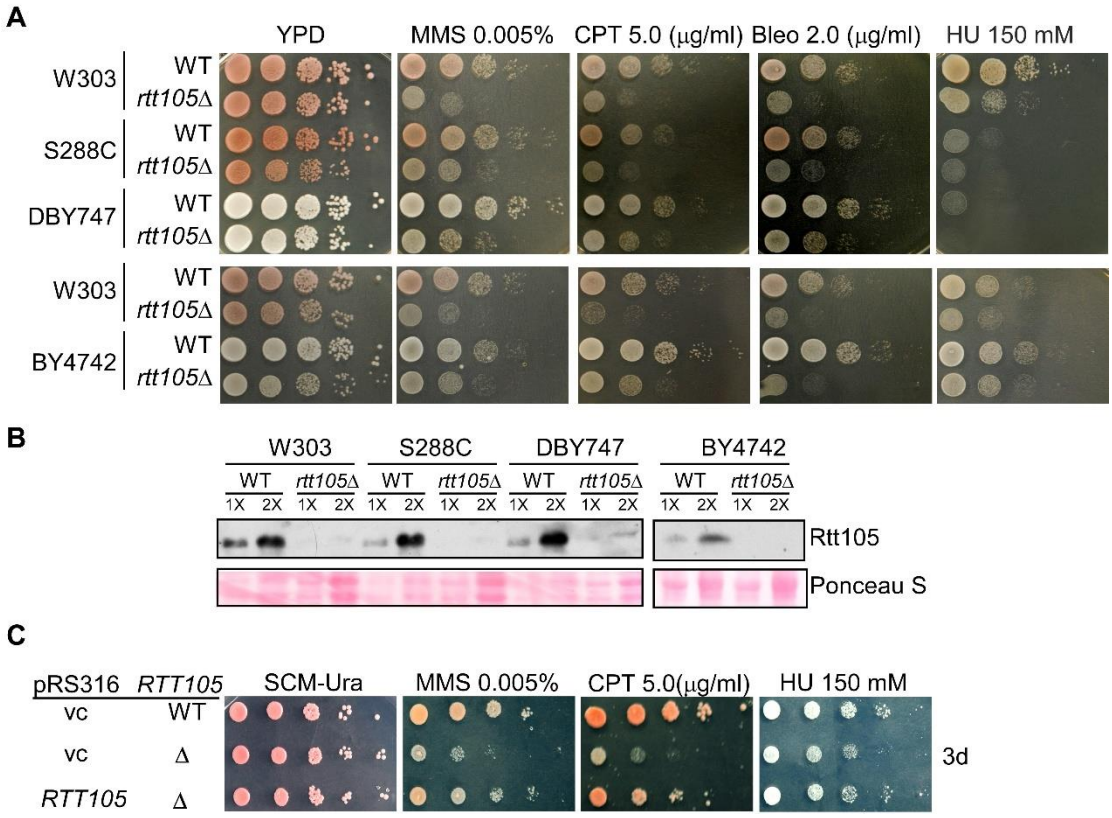
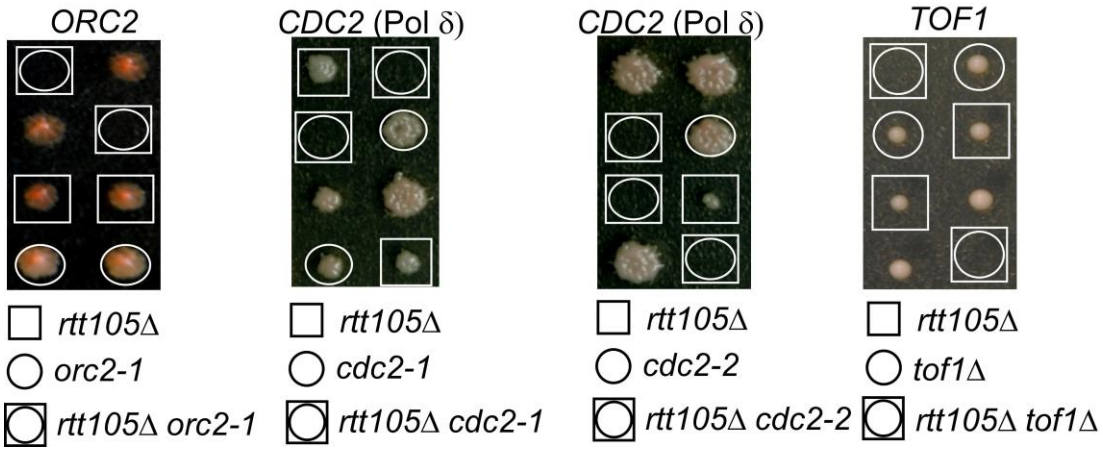
Letter Fig 1



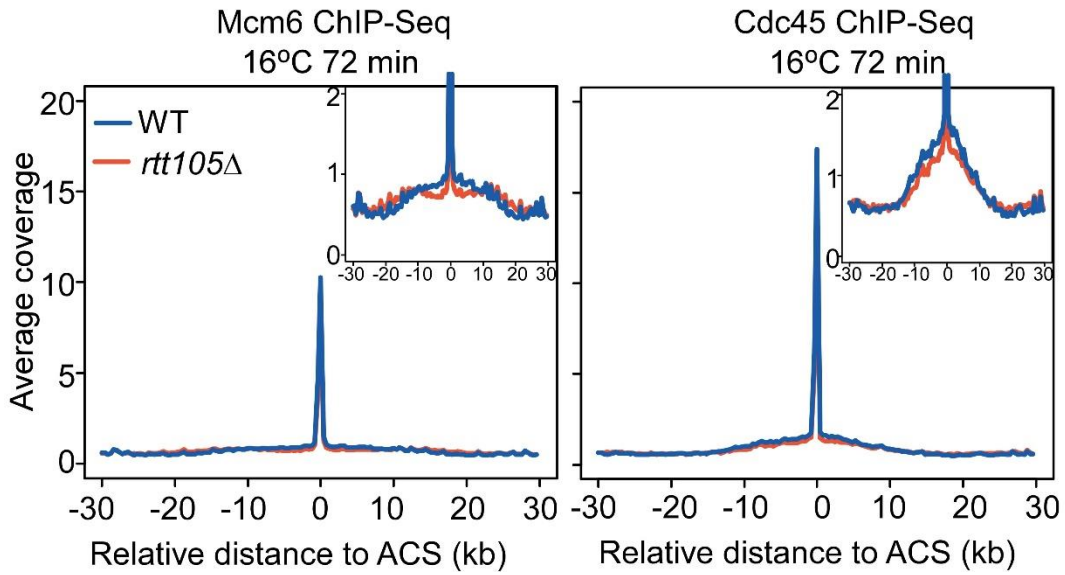
Letter Fig 1. Deletion of RTT105 leads to increased sensitivity to methyl-methane sulfonate (MMS), camptothecin (CPT), hydroxyurea (HU), and bleomycin (Bleo) in several genetic backgrounds. (A) DNA damage sensitivity analysis of *rtt105*Δ mutant cells in various genetic backgrounds. 10-fold serial dilutions of the indicated cells were spotted on YPD control or drug-containing YPD plates. All plates were imaged after 2 or 3 days of incubation at 30°C. **(B)** Western Blotting analysis of *rtt105*Δ mutant cells in various genetic backgrounds. Whole cell extracts of indicated yeast strains were subjected to immunoblotting detected by antibodies against Rtt105. Ponceau S staining was used as a loading control. **(C)** To perform Rtt105 rescue assays, full length Rtt105 under the control of its own promoter was cloned into the pRS316 expression vector and then transformed into *rtt105*Δ (W303) mutant cells. Cells expressing either a vector control (vc) or full length *RTT105* were diluted, as above, and spotted on selective growth media (SCM-Ura) with or without the indicated DNA damage agents.

Letter Fig 2



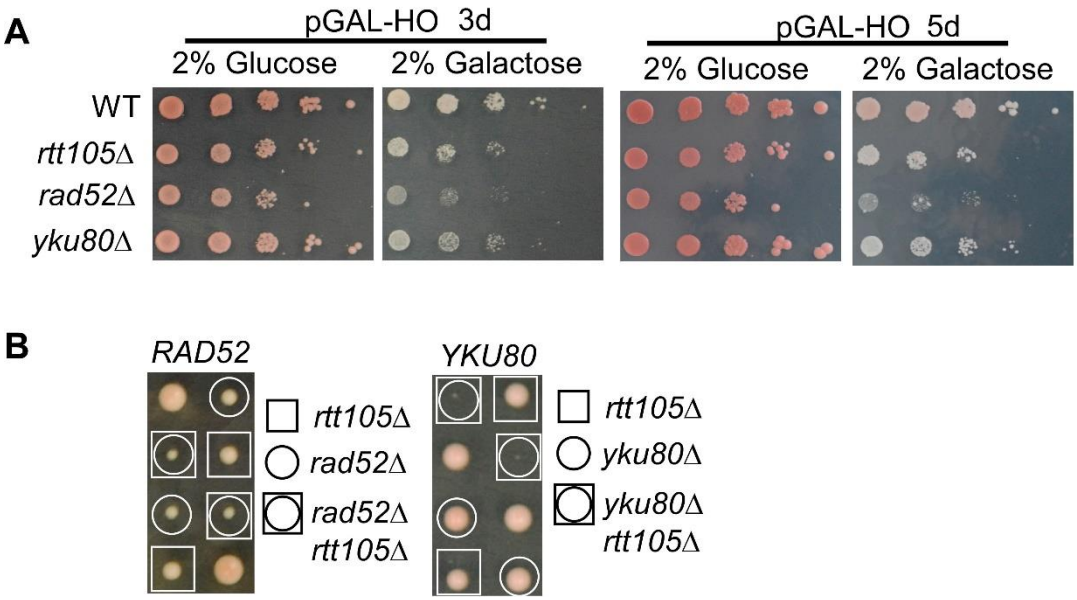
Letter Fig 2. The *rtt105*Δ mutant exhibit synthetic lethal defects with mutations at genes involved in DNA replication. Tetrad analysis of the meiotic progeny of diploid strains was performed in indicated genotypes. Dissected tetrad spores were grown on YPD plates for 3 days before taking pictures.

Letter Fig 3

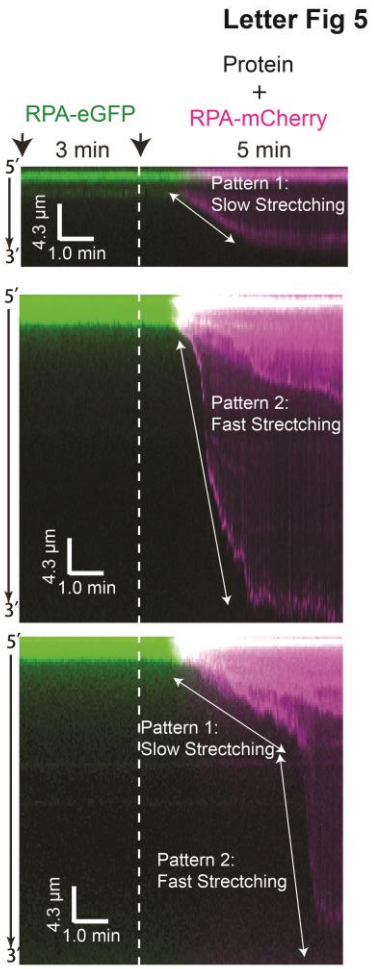


Letter Fig 3. Deletion of *RTT105* does not affect the association of Mcm6 and Cdc45 near ACS during S phase without HU. Mcm6 and Cdc45 ChIP were performed as described in Fig 2 using cells released into 16ºC for 72 min without HU. Under HU conditions, Cdc45 and Mcm6 chromatin association with DNA replication forks are reduced in *rtt105*Δ mutant cells (data not shown).

Letter Fig 4



Letter Fig 4. Rtt105 is important to maintain genome stability. (A) The *rtt105*Δ mutant cells was sensitive to HO-induced double strand breaks (DSBs). Indicated strains harboring p*GAL-HO* plasmid were spotted onto either 2% glucose- or 2% galactose-containing SCM–URA plates in 10-fold serial dilutions. All plates were imaged after 3 to 5 days of incubation at 30°C. **(B)** Tetrad analysis of the meiotic progeny of diploid strains was performed in indicated genotypes. Dissected tetrad spores were grown on YPD plates for 3 days before taking pictures.



Letter Fig 5. Kymograms showing two distinct stretching patterns of RPA-ssDNA molecules. Pattern 1, slow stretching; Pattern 2, fast stretching. An ssDNA molecules exhibit two stretching events in the presence of Rtt105 is shown (bottom panel).

1st Editorial Decision 6th March 2018

Thank you for response letter and proposal for revising your manuscript in response to the comments of our three referees. I have now had a chance to carefully consider them, and I was happy to see that you seem to be in a good position to satisfactorily address the majority of the key concerns raised by myself and the referees. I would therefore like to formally invite you to prepare a revised manuscript along the lines discussed in your response letter. In particular, it would be important to incorporate the five Response Letter figures into the main/EV/Appendix figures as appropriate; and to test cellular Kap95-Rtt105 interactions as well as the effect of reduced temperature as proposed in your answers. Regarding point 3 of referee 2, I appreciate that analyzing Rtt105 roles in RPA accumulation at induced double strand breaks may be the topic of in-depth follow-up investigations, but I nevertheless feel that to adequately answer this point, the data in Response Letter figure 4 should be added to the paper and ideally complemented by some simple assays of rtt105Δ effects on responses to MMS. With these additions, we should be happy to consider a revised version further for The EMBO Journal.

We appreciate all the referees' comments, and point-by-point responses are below.

Referee #1 (Report for Author)

This study by Li et al. addresses the role of the S. cerevisiae Rtt105 protein. They find that Rtt105 physically associates with RPA, a ssDNA binding heterotrimeric complex, and with Kap95, the importin beta subunit that functions in nuclear protein import. They show that Rtt105 directly interacts with RPA and is required for RPA nuclear localization, presumably via mediation of RPA-Kap95 interaction. Since RPA is essential for replication, they find, accordingly, that absence of Rtt105 leads to replication stress sensitivity. Interestingly, they find that when RPA is modified with a nuclear localization signal (bypassing the Kap95 importin system), RPA recruitment at replication forks remains defective, indicating that Rtt105 plays an extra role. Through in vitro ensemble and single-molecule assays they find that Rtt105 promotes/facilitates loading of RPA on ssDNA and detaches from RPA once the latter is bound to ssDNA. They find that treatment of rtt105delta cells with agents that induce replication stress is toxic and leads to genome alterations. They conclude that Rtt105 is important for maintaining genome integrity under these conditions. Overall, this is a well-documented and controlled study and the conclusions are convincing.

Response: We thank the referee for their time and effort in reviewing our manuscript and highly appreciate the very positive comments on this exciting story.

- minor concerns

To fully support the model (Figure 7), could the interaction of Rtt105 with Kap95 in the cytoplasmic fraction but not in the nuclear fraction be confirmed?

Response: We thank the referee for pointing this out. Based on the SGD database, Kap95 is localized to the cytoplasm and nuclear periphery. To test where the interaction of Kap95 with Rtt105 occurs, we used a Bimolecular Fluorescence Complementation (BiFC) assay to visualize protein-protein interactions in living yeast cells (Appendix Fig S5). The C-terminal fragment of Venus fluorescent protein was fused to Rtt105 (Rtt105-Vc155) and The N terminal fragment of Venus was fused to Kap95 or Rfa1 (Kap95-Vn173 or Rfa1-Vn173). Interactions of Kap95 with Rtt105 or Rfa1 with Rtt105 bring the fluorescent fragments within proximity, allowing the reporter protein to form a structure close to the native state and fluoresce when illuminated. We found that the Kap95-Rtt105 signal was more pronounced at the nuclear periphery while the Rfa1-Rtt105 signal was bright within the nucleus (Appendix Fig S5). Based on this result and the referee's comment, we think that it is not appropriate to draw free Kap95 in the nucleus. Therefore, we modified the cartoon accordingly.

We noticed that Rtt105 associates with Rim1 (Table EV1). Since Rim1 is a ssDNA binding protein important for mitochondrial DNA replication, could Rtt105 also be

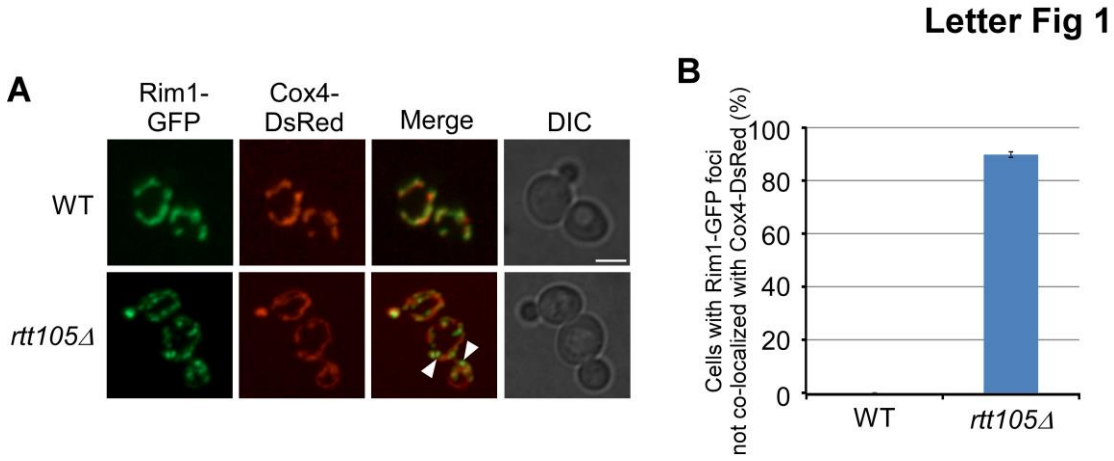
important for mitochondrial genome integrity maintenance?

Response: We thank the referee for highlighting this possibility. To test this idea, we analyzed the impact of *rtt105*Δ on the localization of Rim1-GFP. As expected, the Rim1-GFP fusion protein co-localizes with Cox4-dsRed, a protein known to reside in mitochondria in wild type cells (Letter Fig 1). This localization is compromised in *rtt105*Δ cells, indicating that Rtt105 may also regulate the localization of Rim1 in mitochondria and possibly mitochondrial genome maintenance. I think that a mechanism for Rtt105 function in this process is beyond the scope of the current study. Therefore, we decided to only include the figure in the Letter Figure below, for the benefit of the editor and referees. If the referee thinks that it is more appropriate to include in the Appendix, we would be happy to do this.

To address this concern further, we added the following to the Discussion (Page 18):

"*Rim1 is an ssDNA binding protein important for mitochondrial DNA replication (Van Dyck et al, 1992). Therefore, it would be interesting to determine whether Rtt105 also has a role in regulating Rim1 localization and ssDNA binding in the mitochondria.*"

Letter Fig 1



Letter Fig 1. Rtt105 is important for Rim1's localization in mitochondria. (A) Fluorescence images of cells from wild-type (WT) and *rtt105*Δ cells expressing Rim1-GFP (Rim1-GFP) and Cox4-dsRed (Cox4-DsRed) fusion proteins. Mitochondrial signal was determined by imaging cells expressing the mitochondrial marker Cox4-dsRed. (B) Quantification of cells with Rim1-GFP foci that do not co-localize with the mitochondrial marker. Approximately 100 cells from three independent experiments were quantified and data are represented as mean ± SEM.

Referee #2 (Report for Author)

Through an exhaustive study that involves a large number of *in vivo* and *in vitro* approaches, Li and colleagues report in this work the function of yeast Rtt105; their findings are novel and sound: Rtt105 physically interacts with RPA, escorts it to the nucleus (in part by facilitating the interaction between RPA and its importin) and promotes the loading and mode of binding of RPA at replication forks. According to these functions the absence of Rtt105 causes genetic instability and makes cells sensitive to DNA damaging agents. From these results, the authors conclude that Rtt105 is a chaperone for RPA that is important for genetic stability. The manuscript is well written, concise, and with clear objectives; the experiments have been carefully performed and lead to important conclusions to understand the biology of RPA, an essential and key complex in DNA replication, checkpoint activation and DNA repair. However, some questions remain unclear that should be addressed before publication.

Response: We thank the referee for their time and efforts in reviewing our manuscript and highly appreciate the very positive and constructive comments. We have performed additional experiments to address the referee's concerns, as detailed below.

Major concerns

1. My major concern is the apparent contradiction between the levels of RPA at replicating forks and the rate of DNA synthesis (and cell fitness) under unperturbed conditions. Authors claim in discussion that cells are likely to tolerate low levels of RPA in most chromatin regions, and support this conclusion with the work by Toledo et al. However, these authors reduce the levels of RPA to approximately the half, thereby sensitizing only to HU, which exposes more ssDNA and demands more protection. By contrast, the amount of RPA at both the nucleus (by fluorescence) and chromatin (by ChIP) in rtt105 is barely detectable. If the authors are right they should be able to reduce Rfa1 to similar levels as those shown by rtt105 without affecting viability. Alternatively, the length of ssDNA at forks in rtt105 might be shorter than in the wt, or another protein might protect the forks under conditions of RPA limitation. A candidate is Rad51, which competes with RPA for binding to ssDNA and travels with the fork under unperturbed conditions (González-Prieto et al. 2013; EMBO J).

Response: We understand the referee's concerns. We want to highlight a few observations.

First, possibly due to our inadequate explanation, we may have inadvertently led the referee to believe that RPA is completely gone based on ChIP and immunofluorescence. ChIP assays are good at detecting the relative amounts of a protein at a given chromosome locus. Although we observed a dramatic reduction of RPA binding at replication forks in *rtt105*Δ cells based on ChIP-seq, we do not know

the amount of RPA associated with DNA replication forks in *rtt105*Δ mutant cells under unperturbed conditions. Based on immunofluorescence assays, it is clear that RPA is present in both the cytoplasm and the nucleus in *rtt105*Δ cells, instead of predominantly localized to the nucleus as in wild type cells (Fig 2D-F). Moreover, it is not very clear that the basal (native) levels of RPA are necessary for normal DNA replication in yeast cells. In human cells, it is estimated that RPA is in about 5- to 6-times excess of what is needed to protect ssDNA during normal DNA replication (Toledo et al, 2017). If this is also true for yeast cells it may explain the subtle DNA synthesis phenotype of *rtt105*Δ cells. However, we agree with the referee that it may not be fair to compare RPA levels in *rtt105*Δ cells with the partial reduction of RPA in mammalian cells in Toledo et al.'s work. To address this concern, we modified the text accordingly and discuss this idea and other potential explanations as well (see below and Discussion, Page 16).

Although we did not detect a global reduction in DNA synthesis in *rtt105*Δ cells using BrdU-IP-seq, we did observe several phenotypes of *rtt105*Δ cells supporting the idea that Rtt105 has a role in DNA replication.

First, we show that *rtt105*Δ mutant cells display a relatively smaller colony size and progress more slowly through S phase (by 5-10 min) than wild type cells (Appendix Fig S9B and Appendix Fig S2B). While this defect is relatively mild, we would like to point out that other proteins involved in DNA replication—including Mrc1 (Osborn & Elledge, 2003), Ctf4 (Tanaka et al, 2009; Wang et al, 2010), and Rrm3 (Syed et al, 2016)—induce only mild delays in S phase onset when mutated and yet are very important for the molecular mechanism of copying DNA.

Second, a genome-wide study (Collins et al, 2007) showed that the *rtt105*Δ mutation is synthetic lethal with mutations in genes relevant to DNA replication. We also observed such synthetic defects with mutations in genes involved in DNA replication, including Orc2 and Pol delta (Appendix Fig S8C). All these results indicate that *rtt105*Δ cells have defects in DNA replication under normal growth conditions. Our inability to detect changes in DNA synthesis using BrdU-IP-seq is likely due to the insensitivity of this assay to minor defects in DNA synthesis.

Unfortunately we couldn't establish an effective method to measure the ssDNA length at replication fork regions. We also performed Rad51-ChIP, but we could not detect Rad51 signals at DNA replication origins under unperturbed conditions despite repeated attempts, which is likely due to the fact that Rad51 binds to stalled forks (González-Prieto et al, 2013; Urulangodi et al, 2015). To test the reviewer's idea further, we tested whether the *rtt105*Δ mutation exhibits a synthetic defect with *rad51*Δ. Indeed, we found that *rad51*Δ *rtt105*Δ double mutant cells showed a much more severe growth defect compared to cells of either single mutant (Appendix Fig S9B), raising the possibility that Rad51 may bind ssDNA in *rtt105*Δ cells and compensate for the reduced RPA during normal DNA replication.

In the revised manuscript, we cite this paper and discuss all these ideas (Page 16-17).

We also suggest another possibility: that there exists another RPA chaperone that helps load RPA during normal S phase.

Finally, based on the fact that DNA synthesis is reduced significantly in *rtt105*Δ cells treated with HU, we suggest that Rtt105 is needed more during DNA replication stress when ssDNA is more prevalent.

In addition, a parallel ChIP against another component of the replisome would be a nice control of replisome integrity under unperturbed conditions.

Response: We performed ChIP-Seq analysis of Mcm6 and Cdc45, two components of the active replicative helicase CMG complex, under normal conditions (Appendix Fig S3)**.** We observed that the association of Mcm6 and Cdc45 with DNA replication forks is not compromised to a detectable degree in *rtt105*Δ mutant cells, further supporting our idea that deletion of *RTT105* affects RPA association with DNA replication forks directly.

2. The authors show convincing in vitro results that Rtt105 does not remain at the ssDNA after loading RPA. However, a ChIP assay is missing showing that Rtt105 is not detected in vivo either.

Response: This is a good suggestion, and we have indeed performed Rtt105-ChIP. However, we could not detect Rtt105 at DNA replication origins despite repeated attempts. While this result supports the reviewer's idea, we did not know how to cleanly interpret this "negative" result as we do not have a positive control site for Rtt105 ChIP.

3. In discussion the authors attribute the sensitivity to DNA damaging agents and the accumulation of spontaneous DNA damage to the role of Rtt105 in DNA replication. They should consider the possibility that the sensitivity to DNA damage agents - and part of the accumulation of spontaneous lesions - might be due to a role of Rtt105 in loading RPA at the ssDNA fragments generated upon processing of DNA lesions (a replication-independent function). In this regard, it would further extend the implications of Rtt105 in genetic stability if the authors analyzed RPA accumulation at a HO-induced DSB by ChIP analysis. Indeed, and connected to point 1, how is the checkpoint response to induced DNA damage (e.g., MMS)? RPA is required for Mec1/Ddc2 recruitment and Rad53 activation and therefore a reduction in RPA loading should, in principle, affects this response.

Response: We appreciate the referee's suggestions and insightful thoughts. We agree that we cannot exclude the possibility that the DNA damage sensitivity in *rtt105*Δ mutant cells is due to processing of DNA lesions and/or the DNA damage response. In the revised text, we show that *rtt105*Δ cells are sensitive to HO-induced DSBs (Appendix Fig S9A). Moreover, both *rad52*Δ *rtt105*Δ and *yku80*Δ *rtt105*Δ double mutant cells show a synthetic growth defect (Appendix Fig S9B). Hence, Rtt105 may not be

dedicated solely to DNA replication. We fully agree with the referee that it would be very interesting to determine whether Rtt105 has a role in RPA binding at an HO-induced DSB, and how the *rtt105*Δ mutation affects the DNA checkpoint response. However, we feel that these issues are beyond the scope of the current study. As it stands, the current work contains 7 Figures, 5 Expanded View Figures, 1 Expanded View Table and 9 Appendix Supplemental Figures. We worry that additional data on the role of Rtt105 in DNA damage response and HO-mediated repair would dilute the main message of the current study.

To address this concern, we point out in the Discussion that it would be interesting to determine whether Rtt105 regulates checkpoint activation and whether it has a role in double strand DNA repair (Page 18).

Minor points

- Figures 2 and 6. Include the profile of RPA and BrdU of a whole chromosome (as supplementary information)
Response: We have included these results in Fig EV2E, 2F, and EV5.

- Figure 3. Panels B and C are changed (correct also in the legend and the main text)
Response: We are sorry for this oversight and corrected it in the revised text.

- Figure 4D. Relocate Rfa3 marker
Response: We are sorry for this oversight and corrected it in the revised text.

- Figure 5C, 5D and 5E: Are statistically significant the differences?
Response: Yes, they are. We have put p-values in the revised text.

- Figure 5D: are N=143 and 80 right? (Instead of 99 and 65)
Response: We are sorry that we did not describe this clearly. During the analysis of the ssDNA stretching events, some of the ssDNA molecules exhibit two stretching events (revised Figure 5B, lower panel). Thus, the total number of stretching events is higher than the number of ssDNA molecules. We have described this in more detail in the revised text.

- Legend Fig. EV4 (C): Rtt105 increased the RPA-mCherry binding to ssDNA (?) According to the panel and the main text Rtt105 does not affect this parameter
Response: We are sorry for this oversight and and corrected it in the revised text.

- Legend EV5 (B) refers to Fig 6G (it is 6H)
Response: We are sorry for this oversight and and corrected it in the revised text.

- Page 7 (Appendix Fig S2B should be S2C)
- Page 8 (Appendix Fig S2C should be S2B)

Response: We are sorry for this oversight and and corrected it in the revised text.

- Page 9: the length of ssDNA in the second chase increased ~2.5 and ~4.5 according to Figure 5E (no 1.5 and 5)
Response: We are sorry for this oversight and and corrected it in the revised text.

- Page 10: Fig EV3A should be Fig S1A
Response: We are sorry for this oversight. We have corrected it in the revised manuscript.

Referee #3 (Report for Author)

In this manuscript, the authors show that Rtt105 interacts with and regulates RPA in the budding yeast. Their results convincingly show that Rtt105 interacts with the RPA complex. In rtt105 null cells, the binding of RPA to replication forks is reduced, and genomic instability was increased. Rtt105 is required for the efficient nuclear import of RPA. Rtt105 also stimulates the binding of RPA to ssDNA in vitro. In the DNA curtain assay, Rtt105 enhances the stretching of RPA-bound ssDNA. The authors propose an interesting model in which Rtt105 functions as a chaperon of RPA to facilitate its function in the replication stress response. Although many results in study are quite interesting, the model still needs to be substantiated by additional evidence. Several important questions about the model have to be satisfactorily addressed.

Response: We thank the referee for their time and efforts in reviewing our manuscript. We have worked to address their concerns as detailed below.

Specific comments:
1. The possibility that the reduction in RPA at replication forks in rtt105 cells is caused by defective replication is not convincingly ruled out.

In 2B and 2C, RPA binding to replication forks was reduced in HU treated rtt105 null cells in early S phase. However, in 6A and 6B, BrdU was also reduced at forks under the same condition. It is impossible to exclude the possibility that the defective binding of RPA to forks is due to initiation problems.

Response: We appreciate the referee's suggestion. As the referee states below and as demonstrated in our original manuscript, we have shown that under unstressed growth conditions without HU, DNA synthesis as measured by BrdU IP-seq is not affected to a detectable degree in *rtt105*Δ mutant cells. But, under the same condition, the association of RPA with DNA replication forks is reduced.

In the revised text, we analyzed the binding of Mcm6 and Cdc45, two components of the active replicative helicase CMG complex, at replication forks under unstressed conditions (Appendix Fig S3). We observed that the association of Cdc45 and Mcm6

with DNA replication origins at early S phase is not affected to a detectable degree under unstressed growth conditions, consistent with the BrdU analysis. These results strongly support the idea that the reduced RPA association with DNA replication forks in *rtt105*Δ mutant cells under this unstressed condition is not due to impaired DNA initiation.

I agree with the referee that we can not rule out that the reduction of RPA associated with HU-stalled DNA replication forks is attributable in part to reduced DNA synthesis. To address this concern, we point out that the reduction of RPA at HU-stalled forks in *rtt105*Δ mutant cells is likely due to a lack of RPA chaperoning activity as well as subsequently impaired DNA synthesis (Page 17).

In 2D and 2E, RPA binding to forks was clearly reduced in rtt105 cells at 16C. However, in 6C and 6D, BrdU incorporation at forks was not defective. Do these results suggest that the role of Rtt105 in enhancing RPA binding is not required for replication? If the role of Rtt105 is specific to the response to genomic instability, the assays for genomic instability should be done at 16C because RPA binding is clearly defective under this condition (2D and 2E). The assays in 6E-G were done at 25C.

Response: We thank the referee for this observation. As pointed out in our response to referee #2, while overall DNA synthesis as detected by BrdU-IP-seq is not affected to a detectable degree, the *rtt105*Δ mutant cells exhibit a mild but consistent slow S phase progression, suggesting that Rtt105 is needed for normal DNA replication. Supporting this idea, we and others observed that the *rtt105*Δ mutant exhibits synthetic defects with mutations in genes involved in DNA replication (Appendix Fig S8C) (Collins et al, 2007). As detailed in our response to Point 1 of referee #2, we offer several explanations for the apparent discordance between the reduction of RPA binding at DNA replication forks and apparently normal overall DNA synthesis as detected by BrdU incorporation in *rtt105*Δ mutant cells under unstressed conditions.

To address the referee's specific concern further, we would like to point out that it is important to use 16°C to slow down DNA synthesis in order to monitor the association of DNA proteins with DNA replication forks genome-wide without using HU (Aparicio et al, 1997). According to previous reports, while DNA replication forks progress more slowly on average than their maximal rate, late origins still fire and cells proceed through the cell cycle, suggesting that cells do not experience a detectable DNA replication stress at 16°C (Aparicio et al, 1997; Yu et al, 2014). Therefore, we adopted this condition for our ChIP-seq analysis. Furthermore, we also performed Rfa1 ChIP-qPCR under unperturbed conditions at 25°C (Fig 2F). We observed that RPA binding at replication forks under this condition is also reduced (Fig 2F). Therefore, the reduction of RPA binding to unstressed DNA replication forks was observed in cells growing at both 16°C and 25°C.

To address this concern even further, we have performed dot assays and monitored

cell growth at 16°C. We found that the *rtt105*Δ cells also exhibit a minor growth defect and increased sensitivity to DNA damaging agents as at 25°C (Appendix Fig S2A).

The experiments done at 25C are somewhat confusing. In 2F, the binding of RPA to forks was clearly reduced in rtt105 cells. However, cell cycle progression was not affected under this condition (S1A). In 6E and 6F, DNA damage accumulated in these cells and the checkpoint was activated. Do these results suggest that the reduction of RPA binding in rtt105 cells does not matter for replication? Is the genomic instability and checkpoint activation in rtt105 cells too weak to slow down S phase? If so, the functional phenotypes of rtt105 cells are quite weak - this is a problem for the overall impact of the paper.

Response: We appreciate and understand the referee's concern. As discussed above, the effect of *rtt105*Δ on DNA synthesis at a global level is subtle under unstressed growth conditions. We would also argue that the significance of a molecule's role in a given process is not necessarily correlated to the functional phenotype of a null mutant under a specific condition (see below). Importantly, we present multiple lines of evidence supporting the idea that Rtt105 does have a novel role in DNA replication (see also discussion in response to referee 2).

First, we show that *rtt105*Δ mutant cells display a relatively smaller colony size and progress more slowly through S phase (by 5-10 min) than wild type cells (Appendix Fig S9B and Appendix Fig S2B) in revised text. While this defect is relatively mild, we would like to point out that other proteins involved in DNA replication—including Mrc1 (Osborn & Elledge, 2003), Ctf4 (Tanaka et al, 2009; Wang et al, 2010), and Rrm3 (Syed et al, 2016)—induce only mild delays in S phase onset when mutated and yet are very important for the molecular mechanism of copying DNA.

Second, a genome-wide study (Collins et al, 2007) showed that the *rtt105*Δ mutation is synthetic lethal with mutations in genes relevant to DNA replication. We also observed such synthetic defects with mutations in genes involved in DNA replication, including Orc2 and Pol delta (Appendix Fig S8C).

In the revised manuscript we offer several potential explanations for the subtle phenotype of the *rtt105*Δ mutant under unstressed conditions. First, it is possible that the reduced amount of RPA at unstressed DNA replication forks in *rtt105*Δ cells is not sufficient to cause a global defect in DNA synthesis that can be detected by BrdU incorporation. Consistent with this idea, in human cells, it is estimated that RPA exceeds ssDNA by 5- to 6-fold under normal replication conditions (Toledo et al, 2017). If this holds true for yeast PRA and ssDNA under normal replication conditions, then it may explain the mild DNA synthesis defects observed in *rtt105*Δ mutant cells under unstressed conditions. Moreover, it is possible that another RPA chaperone contributes to the delivery of RPA under normal growth conditions. This is not a far-fetched possibility. For example, histone chaperone CAF-1 is critical for DNA replication coupled nucleosome assembly from yeast through humans, and yet yeast

cells lacking CAF-1 exhibit only a minor growth defect because two other histone chaperones, Rtt106 and FACT, compensate (Hammond et al, 2017; Yang et al, 2016). Furthermore, as suggested by referee #2, it is possible that Rad51 complements the reduced levels of RPA in *rtt105*Δ mutant cells given that we observe a synthetic defect with *rad51*Δ (Appendix Fig S9C). Finally, we suggest that Rtt105 may play a more important role in DNA replication under replication stress. Supporting this idea, we found that that the replication defects in *rtt105*Δ as measured by BrdU incorporation are much more severe under stress (Fig 6B). Therefore, we propose that Rtt105 plays a prominent role in regulating RPA and DNA replication under replication stress conditions when more ssDNA is exposed.

Finally, I would like to emphasize that it is important for cells to deal with replication stress. DNA replication stress can be caused by a variety of internal events and external agents that impede normal replication progression, including collisions between the DNA replication and gene transcription machineries, DNA damaging agents, and oncogene activation in precancerous lesions in human cells, for example. Therefore, cells have many genes devoted to dealing with replication stress. Our results indicate that Rtt105 is important for regulating the function of RPA, an absolutely crucial protein, during DNA replication stress. In the revised manuscript we discuss these points and emphasize the role of Rtt105 in replication stress (Page 16-17).

2. The results in Fig. 3 clearly show that the nuclear import of RPA is impaired in rtt105 cells. However, there is no data showing that this specific function of Rtt105 is important for the function of RPA. It is necessary to separate this function of Rtt105 and the other functions in RPA-ssDNA regulation.

Response We agree with the referee that it would be interesting to determine the contribution of Rtt105 to RPA nuclear import versus RPA binding to ssDNA. To address this concern, we attempted to map the Rtt105 binding site for Kap95. Unfortunately, we have not been able to identify mutations that affect Kap95 binding but not RPA binding. In hindsight, this is not surprising because it is likely that Kap95-Rtt105-RPA forms a trimer and that complex formation depends on each component. However, I would like to stress that because RPA binding to ssDNA at DNA replication forks occurs in the nucleus, it is expected that a defect in RPA nuclear import will necessarily affect RPA binding at replication forks. Therefore, our original hypothesis was that the reduced RPA binding to DNA replication forks in *rtt105*Δ mutant cells is due to the nuclear import defect. Surprisingly, we observed that expression of an RPA fusion protein that contains a strong NLS signal, while bypassing the requirement of Rtt105 for RPA nuclear localization, cannot rescue the RPA binding defect in *rtt105*Δ mutant cells. These results suggest that Rtt105 has a role in loading RPA to DNA replication forks. Because this novel function has not been observed in other known RPA binding proteins, we decided to focus our studies on this unexpected function of Rtt105. At the same time, we conclude that Rtt105 contributes to both RPA nuclear import and RPA

loading at ssDNA substrates.

3. Several interesting observations were made in the in vitro ssDNA binding EMSAs and DNA curtain assays. However, it is unclear whether and how these observations can be related to each other. In the ssDNA binding assay, only one short ssDNA oligomer (30 nt) was tested. It is not clear whether the effects of Rtt105 on RPA are dependent on the length of ssDNA. In the DNA curtain assay, the binding of RPA-mCherry to ssDNA did not appear to be stimulated by Rtt105, which is not quite consistent with the EMSA results. If the stretching of RPA-ssDNA is distinct from the enhanced RPA binding to ssDNA, how can these functions be separated? The Rtt105 EL is defective for multiple functions and cannot distinguish these possibilities. After all, we still don't know which of the functions of Rtt105 is important for the phenotypes of rtt105 null cells.

Response: We thank the referee for these observations and comments.

We may have been unclear in the original manuscript about the relationship between the *in vitro* EMSA result and ssDNA curtain assay result. Based on the EMSA analysis, RPA binds more efficiently to ssDNA in the presence of Rtt105 than in the absence of Rtt105. This could be due to either an increased binding affinity of RPA for ssDNA, or altered conformational changes in the presence of Rtt105. We therefore performed an orthogonal set of experiments: our ssDNA curtain assays support the second possibility. Therefore, we suggest that Rtt105 alters the conformation of RPA so that RPA can efficiently bind to ssDNA *in vitro*. This conclusion is also supported by our *in vivo* results showing that the association of RPA with ssDNA at DNA replication forks is reduced. Moreover, it is also supported by the new experiment suggested by the referee, as described below.

To answer the referee's question about whether ssDNA length affects the ability of Rtt105 to promote RPA binding to ssDNA *in vitro*, we performed a series of EMSAs using three different lengths of oligodeoxythymidine (oligo (dT), 17nt, 23nt, and 30nt) and compared the effect of Rtt105 on ssDNA binding (Appendix Fig S6). We chose to use these short oligo (dT)s because each short oligo allows one RPA trimer to bind, thereby avoiding the complication of cooperative binding from a second RPA complex. Moreover, it has been shown using these short oligos that yeast RPA engages ssDNA via at least two binding modes: with three OB fold domains of Rfa1 contacting 12-23 nt of ssDNA, and with three OB fold domains of Rfa1 and one OB fold domain of Rfa2 contacting 23-27nt ssDNA (Bastin-Shanower & Brill, 2001; Kim et al, 1994). Consistent with published results (Bastin-Shanower & Brill, 2001), we observed that the binding constant for RPA in the absence of Rtt105 ranges from $0.11X10^8$ M^{-1} for oligo(dT)17 to approximately 0.34×10^8 M^{-1} for oligo(dT)30. Remarkably, Rtt105 promotes the binding of RPA to all of these different lengths of ssDNA substrates (Appendix Fig S6). Importantly, when normalized against RPA binding to the corresponding length of oligo(dT) in the absence of Rtt105, we observed that Rtt105 stimulated RPA binding to

(dT)23 and (dT)30 to a similar degree (14.9-fold and 15.4-fold). In contrast, the stimulatory effect for the oligo(dT)17 was much smaller (3.1-fold) (Appendix Fig S6). These results suggest that Rtt105 changes the ssDNA binding mode of RPA, likely by facilitating RPA to adopt an extended conformation.

Moreover, we show that all these effects depend on the Rtt105-RPA interaction because the Rtt105 EL mutant, which cannot bind RPA *in vitro*, does not exhibit any of these effects on RPA. Taken together, these results strongly support our conclusion that in addition to mediating nuclear import of RPA, Rtt105 has a role in loading RPA onto ssDNA.

We have included these results and rewritten the Results and Discussion to make it clear that both the EMSA and ssDNA curtain assay indicate that Rtt105 can alter the mode of RPA binding ssDNA.

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2nd Editorial Decision 22nd June 2018

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original reviewers, and I am happy to inform you that all three of them are generally satisfied with the revisions and improvements to the paper. Referee 2 still retains some specific concerns, which I like to ask you to respond to and address in a final round of minor revision.

REFEREE REPORTS

Referee #1:

To the opinion of this reviewer, the authors have satisfactorily replied to the initial concerns and have done a decent job further improving the clarity of the manuscript.

Referee #2:

Most of my major concerns have been addressed, and the final version provides sufficient support to the major conclusions, which are sound for the genome dynamics field. However, some points should still be clarified in the text.

- My concern about the fact that rtt105 is listed as inviable is not that the authors worked with a strain that expressed Rtt105, but that the strain was viable because a suppressor had been selected during its construction. To discard this the author need to disrupt RTT105 in a diploid and confirm that the dissected spores are 4:0 for viability. Maybe they did it this, but it is unclear from the text if they disrupted RTT105 in a haploid or diploid strain.
- Even though it is clear from the new results and previous ones that rtt105 display replicative defects under unperturbed conditions, it is still chocking to me how subtle they are considering the dramatic loss of RPA by ChIP. I am aware that this can be due to a loss of efficiency of the ChIP to detect a protein below some levels (an argument that should be included). I disagree with two of the explanations that the authors provide: 1) an excess of RPA relative to the amount needed to protect forks, and 2) an alternative RPA chaperone to delivery RPA in rtt105. Regardless of a putative excess of RPA or the existence of an alternative chaperone, the fact is that there is little RPA at the fork. Authors could consider the possibility that short ssDNA stretches to be relatively stable in the absence - or with little - RPA, which would be consistent with the fact that rtt105 is particularly important in response to HU o MMS, which generate longer ssDNA fragments.
- In discussion, it is not explained why Rad51 could protect in the absence of RPA and include some reference.
- ChIPs Figures EV2B and C, and Figure 3D. A plot of 3 technical replicates is shown, and the statistical analyses are done with these 3 values. This discards that the changes were due to variability in the qPCR amplification, which is unlikely because qPCR are highly accurate using the same biological samples. The relevant data and statistic analyses must be done with the independent experiments to assess how reliable and reproducible the enrichments are. The authors have these data and therefore they must be included.

Referee #3:

The authors have significantly improved the manuscript. I support the acceptance of this manuscript for publication.

We appreciate all the referees' comments, and point-by-point responses are below.

Referee #1:

To the opinion of this reviewer, the authors have satisfactorily replied to the initial concerns and have done a decent job further improving the clarity of the manuscript.

Response: We thank the referee for their time and effort in reviewing our manuscript and highly appreciate the very positive comments on this exciting story.

Referee #2:

Most of my major concerns have been addressed, and the final version provides sufficient support to the major conclusions, which are sound for the genome dynamics field. However, some points should still be clarified in the text.

Response: We thank the referee for their time and efforts in reviewing our manuscript and highly appreciate the very positive and constructive comments. We have modified the discussion to address the referee's concerns, as detailed below.

- My concern about the fact that rtt105 is listed as inviable is not that the authors worked with a strain that expressed Rtt105, but that the strain was viable because a suppressor had been selected during its construction. To discard this the author need to disrupt RTT105 in a diploid and confirm that the dissected spores are 4:0 for viability. Maybe they did it this, but it is unclear from the text if they disrupted RTT105 in a haploid or diploid strain.

Response: We are sorry that we didn't understand the referee's request in the last revision. The *rtt105*Δ strain was constructed using standard method in haploid cells in the beginning. To confirm the viability, we crossed the *rtt105*Δ strain with wild-type haploid, and then performed tetrad dissection analysis. If there is a suppressor as proposed by the referee, one would expect that about 50% *rtt105*Δ spores would be lethal when the suppressor did not co-segregate with *rtt105*Δ. This is not what we observed from many tetrad dissections in this study. Therefore, we think that it is not necessary to delete *RTT105* from a diploid strain.

- Even though it is clear from the new results and previous ones that rtt105 display replicative defects under unperturbed conditions, it is still chocking to me how subtle they are considering the dramatic loss of RPA by ChIP. I am aware that this can be due to a loss of efficiency of the ChIP to detect a protein below some levels (an argument that should be included). I disagree with two of the explanations that the authors provide: 1) an excess of RPA relative to the amount needed to protect forks, and 2) an alternative RPA chaperone to delivery RPA in rtt105. Regardless of a putative excess of RPA or the existence of an alternative chaperone, the fact is that there is little RPA at the fork. Authors could consider the possibility that short ssDNA stretches to be relatively stable in the absence - or with little - RPA, which would be consistent with the fact that rtt105 is particularly important in response to HU o MMS, which generate longer ssDNA fragments.

Response: We appreciate the referee's suggestions and insightful thoughts. We have modified the discussion to include additional possibilities proposed by the referee (Page 16). In fact, we have attempted to covey a similar message, but appeared to get lost in the last version.

- In discussion, it is not explained why Rad51 could protect in the absence of RPA and include some reference.

Response: We thank to the referee's suggestion. We have modified the discussion and included the reference (Page 17)

- ChIPs Figures EV2B and C, and Figure 3D. A plot of 3 technical replicates is shown, and the statistical analyses are done with these 3 values. This discards that the changes were due to variability in the qPCR amplification, which is unlikely because qPCR are highly accurate using the same biological samples. The relevant data and statistic analyses must be done with the independent experiments to assess how reliable and reproducible the enrichments are. The authors have these data and therefore they must be included.

Response: We are sorry for this oversight. We have now include average plots and statistical analysis from independent ChIP experiments in the revised figures.

Referee #3:

The authors have significantly improved the manuscript. I support the acceptance of this manuscript for publication.

Response: We thank the referee for their time and effort in reviewing our manuscript and highly appreciate the very positive comments on this exciting story.

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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1. Data

The data shown in figures should satisfy the following conditions:

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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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Is there an estimate of variation within each group of data?	Yes
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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Yes.
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22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No