EMBO reports

# Rad9/53BP1 protects stalled replication forks from degradation in Mec1/ATR-defective cells

Matteo Villa, Diego Bonetti, Massimo Carraro, and Maria Pia Longhese

**Review timeline:**

| | |
|---|---|
| Submission date: | 28 July 2017 |
| Editorial Decision: | 28 August 2017 |
| Revision received: | 13 November 2017 |
| Editorial Decision: | 27 November 2017 |
| Revision received: | 29 November 2017 |
| Accepted: | 8 December 2017 |

Editor: Esther Schnapp

## Transaction Report:

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

1st Editorial Decision 28 August 2017

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, the referees acknowledge that the findings are potentially novel and interesting. However, they also point out that the study would need to be strengthened, and that several controls should be added. Together, they have a number of suggestions for how the work should be improved, and I think that all of them make sense and should therefore be addressed (except may be for the last major comment by referee 3, which is optional).

Given these constructive comments, we would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

 

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

In this manuscript, Villa et al. describe a new role for Rad9 in promoting survival and replication completion of Mec1-defective cells facing a replication stress imposed by hydroxyurea (HU). Based on genetic analyses carried out with several specific mutants, the authors suggest that the main role of Rad9, in complex with Dpb11, is to protect stalled replication forks from extensive DNA resection promoted by Dna2/Sgs1 (but not Exo1) and facilitated by the chromatin remodeller Fun30 and the scaffold protein Slx4. They use a specific background for Mec1 deficiency (mec1-100) combined with the loss of Rad9 or with a specific mutant of Sgs1 (sgs1-G1298R) that escapes inhibition from Rad9 (previously described in another paper from the same group). Combining sgs1-G1298R or rad9Δ with this mec1 deficiency strongly sensitize cells to HU, impairs replication completion and promotes the formation of single stranded DNA (ssDNA) at and nearby replication origins.
Overall, this story is very interesting, this manuscript is well written, the data are of good quality and it should be of interest for a broad audience in the field of genome stability. However, I may not completely agree with some of the conclusions and additional experiments may help to strengthen the message proposed by the authors.

Major concerns
1. The authors only use a specific Mec1 background (mec1-100). Can we generalize their findings about this protective role of Rad9 to other Mec1-defective cells (either mec1Δ sml1Δ with very low HU doses or other hypomorphic mutants) or even to other checkpoint mutants (Mrc1Δ or Rad53 hypomophic for instance)?

2. The authors propose that an increased resection by Dna2/Sgs1 (but not Exo1) at stalled forks is responsible for the poor HU survival and the replication deficiency of mec1-100 rad9Δ cells. Even if the mec-100 sgs1Δ double mutant already gives a strong HU sensitivity (Cobb et al., Genes Dev, 2005), it could be interesting to see whether sgs1 deficiency might somehow rescue the phenotype of mec1-100 rad9Δ cells. This would again argue that Dna2-Sgs1 dependent resection is toxic in mec1-100 rad9Δ cells.

3. The authors argue several times that "This Rad9 protective function is independent of checkpoint activation". However, rad9Δ mec1-100 cells are more sensitive to HU than sgs1-G1298R mec1-100 cells and display an undetectable Rad53 phosphorylation. The experiment with the rad9-STAA allele (defective in interacting with Dpb11) supports a checkpoint-independent checkpoint of Rad9 since rad9-STAA mec1-100 cells are much more sensitive than mec1-100 cells while Rad53 activation is similar in both strains. However, at 3mM HU in Figure 4A, rad9Δ mec1-100 cells are more sensitive than rad9-STAA mec1-100, raising the possibility that the phenotypes observed in rad9Δ mec1-100 are partially dependent on a defect in checkpoint activation, in addition to the role

 

of the Rad9-Dpb11 complex in antagonizing DNA resection. A role for Rad9 in promoting checkpoint signalling could also explain why the phenotypes of the rad9Δ mec1-100 are more dramatic than those of the sgs1-G1298R mec1-100 cells. Finally, the mec1-100 background already constitutes a particular checkpoint deficiency so the authors may want to be more precautious when stating that the Rad9 effect is checkpoint-independent.

4. Related to point 3. Given the known anti-checkpoint role of Slx4 (Ohouo, Nature, 2013) in antagonizing Rad9-Dpb11 dependent signalling, a WB assessing Rad53 activation in the strains used in figure 4C could be informative. Indeed, Slx4 deficiency suppresses the HU sensitivity of mrc1Δ cells, likely by restoring Rad53 activation (Ohouo, Nature, 2013). It is conceivable that Slx4 deficiency similarly supresses the HU sensitivity of mec1-100 cells by restoring a "WT" Rad53 activation.

5. The authors propose that the "hyperstabilization of Rad9-Dpb11 interaction suppresses the HU hypersensitivity of mec1-100 cells".
This is based on the known competition between Slx4 and Rad9 for Dpb11 (Ohouo, Nature, 2013). However, I am not aware that such a competition exists between Fun30 and Rad9 for Dpb11 binding, which is presented as the rationale for the use of Fun30 and Slx4 mutants. Could the authors eventually show (by coIP) that the interaction between Rad9 and Dpb11 is actually increased in the absence of Slx4 (as expected) or in the absence of Fun30? On the contrary, is the interaction between Slx4 and Dpb11 increased in the rad9-STAA mec1-100 cell lines?
Moreover, the interaction between Slx4 and Dpb11 (but not between Dpb11 and Rad9) is strongly dependent on Mec1 after DNA damage (Liu, JCB, 2017). What about the HU-induced Slx4 phosphorylation in a mec1-100 background? Is it decreased thus weakening the Dpb11-Slx4 interaction? If yes, the Dpb11-Rad9 interaction might be stabilized in mec1-100 cells and represent a strong block to DNA resection, which might contribute to the dependency of mec1-100 cells on Rad9?

6. I have several concerns about Figure 5 and the conclusions the authors draw from their data.
6a: Why the level of ssDNA at ARS607 (and at 0.5kb) does not decrease over time in WT cells, as would be expected from the Rpa1 ChIP data showing a decrease in Rpa1 binding over time?
6b: Why is there no significant ssDNA generation in WT cells beyond 1,7kb from the ARS over time? I would expect that as replication proceeds away from the ARS, ssDNA generation would be detectable at later times points at these loci.
6c: How can the authors be sure than the ssDNA they detect originates from resection rather than uncoupling between the replicative helicase and the polymerase(s)?
Do the authors think that this is due to the resection of the nascent strands and in this case, lagging and/or leading strand? Or could it be the consequence of a DSB at collapsed forks followed by a "classical" DNA end resection? Have the authors looked at whether there is an increase in DSBs in the double mutants? One source of collapsed forks could be the action of a structure-specific endonuclease (such as Mus81-Mms4) at unprotected stalled forks.
6d: why does ssDNA accumulate over time both at the ARS (without reaching a plateau) and at distant loci (3kb and 5kb) in the double mutants rad9Δ mec1-100 and sgs1-G1298R mec1-100? Could this be actually explained by the specific and continuous resection of the Okasaki fragments on the lagging strand by Dna2/Sgs1 in the double mutants? But if this is the case, resection could seem slow?
6e: If this ssDNA increase is due to resection, one could expect that this could be reversed when combined with a defect in resection. Can the authors analyse ssDNA generation (eventually combined with Rpa1 ChIP analysis) in a dna2-1 mec1-100 rad9Δ background and see whether it is decreased compared to mec1-100 rad9Δ?
6f: Is it possible to more directly address resection by BrdU ChIP to see whether neosynthetized DNA is actually resected over time?
To conclude, I think this section needs clarification and that a model would help the readers to understand how resection happens in mec1-100 Rad9Δ cells.

Minor comments

1. Figure S1 should be incorporated in Figure 5.

2. Visually, I would invert (upside down) the panel D of the Figure 2 with the high molecular

 

weights DNA species at the top of the gel (and mentioning with an arrow these hMW DNA).

Referee #2:

Rad9 and its human ortholog, 53BP1, have well documented roles in preventing extensive resection at DNA double-strand breaks (DSBs). However, the role of Rad9 is preventing resection at stalled replication forks has not previously been addressed. In this study, the authors present data supporting a role for Rad9 in protecting stalled forks from excessive degradation when the replication checkpoint is partially compromised. These studies make use of the mec1-100 hypomorphic allele, which is partially defective for the intra-S phase checkpoint and confers sensitivity to HU. The authors show that the sgs1-G1298R, a gain of function allele that is resistant to Rad9-mediated inhibition of resection at DSBs, synergizes with mec1-100, and this effect is also seen for the rad9 mutation. The most compelling data to support the proposal that Rad9 prevents extensive resection at stalled forks is presented in Fig 5, where the authors show increased ssDNA in the vicinity of ARS607 in checkpoint compromised cells defective for Rad9 or with the sgs1-G1298R mutation.

Given the current interest in replication fork stability the work it timely and suggests the role of Rad9 in suppressing resection is not limited to DSBs. There are some experimental concerns that need to be addressed:

The data presented in Fig 2D do not make sense. The nascent DNA labeled with BrdU should be chased to high molecular weight during the -HU recovery in the WT strain, instead it stays as low molecular weight intermediates. Is the gel flipped?

If the inviability of mec1-100 sgs1-G1298R cells is due to excessive resection, surely one would expect that reducing resection with the dna2-1 mutation would rescue the mec1-100 sgs1-G1298R hypersensitivity to HU (Fig 3). Although dna2-1 does partially suppress the hyper-HU sensitivity of the mec1-100 rad9 mutant it should also suppress mec1-100 sgs1-G1298R. Given the complication of asking for survival as a read out and the potential of other effects of exo1 and dna2-1 on replication and recovery from arrest, I recommend the authors monitor ssDNA formation at ARS607 in mec1-100 sgs1-G1298R exo1, mec1-100 sgs1-G1298R dna2-1 and mec1-100 rad9 dna2-1 strains to ensure the increase in ssDNA is really due to resection by Dna2 nuclease. I suspect there could be some contribution from Exo1 in the mec1-100 rad9 strain because Rad53 activation in response to HU is abolished in these cells and Exo1 phosphorylation by Rad53 negatively regulates its activity.

The mec1-100 mutant has a delayed checkpoint response as measured by Rad53 activation. Is the amount of Rad9 bound to stalled forks when cells are treated with HU decreased in the mec1-100 and mec1-100 sgs1-G1298R mutants? Rad9 binding could be assessed by ChIP at ARS607.

The authors use the mec1-100 allele to partially compromise the replication checkpoint. Is fork degradation seen in mec1 null or chk1 mutants?

Does the increase in ssDNA at replication forks in mec1-100 sgs1-G1298R or mec1-100 rad9 cells cause more fork collapse?

Minor comments
P. 6: Sgs1 is not the only yeast RecQ helicase, Hrq1 is also in the RecQ family.
Fig 4C: The spot assays are messy; are the large colonies on 20 and 25 mM HU plates contaminants or suppressor mutants?

Referee #3:

The manuscript by Villa and colleagues describes a detailed analysis of the protection of compromised replication forks by a checkpoint independent function of Rad9/53BP1. They present arguments indicating that Rad9 protective function act through the inhibition of the Sgs1/Dna2 mediated resection independently of its checkpoint function. They also provide genetic arguments suggesting that Rad9 is recruited by Dpb11 at compromised replication forks.

 

In general, this work is of high quality, the manuscript is well written and this paper addresses mechanistic questions that are of great interest to researchers in the replication field.
The results presented are based on the analysis of a combination of genetic interaction with previously characterized mutants. However most of the mutants used haven't been previously characterized for their behavior in response to HU and this should be systemically done in this paper. I thus consider that some controls (listed below) are lacking at present.
Major comments:
- Figure 1F: The double rad9 sgs1-G1298R is missing.
- Figure 2B: The single rad9, sgs1-G1298R and the double rad9 sgs1-G1298R should be shown.
- Figure 2D: The high molecular weight products expected in WT strains appear at the bottom of the blot. Isn't this blot flipped upside down?
- Figure 3A: The exo1 sgs1-G1298R control is missing.
- I also have a concern on the conclusion drawn from panel 3 B and C. Although I agree that the lack of Rad9 requires Dna2 to exacerbate the HU sensitivity of the mec1-100 mutant, I don't think that the authors can say the same for the expression of Sgs1-G1298R. Indeed, they observed that the sgs1- G1298R mec1-100 mutant is less sensitive than the sgs1-G1298R dna2-1 mec1-100 and as sensitive than the dna2-1 mec1-100. This, I think only suggests that Sgs1-G1298R and Dna2-1 act in the same pathway and that the dna2-1 mutant is either more severely affected or has an additional function.
- Figure 4B: Rad53 phosphorylation should also be tested in the rad9-Y798A and rad9-STAA single mutants.
- I could also be worth testing the whole set of single and double mutants for their progression through S phase upon HU treatment and see whether Δfun30 and Δslx4 restores or not the Rad53 phosphorylation in mec1-100 mutants upon HU.

Minor comments:
Sgs1 has a broad range of contributions at replication forks, which often renders it difficult to determine the exact interplay of the partially redundant pathways, that enable fork restart, checkpoint activation. Notably in this manuscript, understanding why both deletion of SGS1 and a uncontrolled resection by the sgs1-G1298R mutant both lead to increased HU sensitivity in response to HU in mec1-100 mutant possibly requires a background on Sgs1 function at replication forks. I thus suggest the authors to add a paragraph on this in the introduction to help non-specialized readers.
For the same reason, the rational of testing mus81 mutants and an interpretation on the absence of synergism in the mus81 sgs1-G1298R should be given p6.

I would like to emphasize the clarity of this manuscript and the high quality of the data presented. I will be happy to see it published soon.

1st Revision - authors' response 13 November 2017

Response to reviewer's comments

*Referee #1:*

*In this manuscript, Villa et al. describe a new role for Rad9 in promoting survival and replication completion of Mec1-defective cells facing a replication stress imposed by hydroxyurea (HU). Based on genetic analyses carried out with several specific mutants, the authors suggest that the main role of Rad9, in complex with Dpb11, is to protect stalled replication forks from extensive DNA resection promoted by Dna2/Sgs1 (but not Exo1) and facilitated by the chromatin remodeller Fun30 and the scaffold protein Slx4. They use a specific background for Mec1 deficiency (mec1-100) combined with the loss of Rad9 or with a specific mutant of Sgs1 (sgs1-G1298R) that escapes inhibition from Rad9 (previously described in another paper from the same group). Combining sgs1-G1298R or rad9D with this mec1 deficiency strongly sensitize cells to HU, impairs replication completion and promotes the formation of single stranded DNA (ssDNA) at and nearby replication origins.*
*Overall, this story is very interesting, this manuscript is well written, the data are of good quality and it should be of interest for a broad audience in the field of genome stability.*
*However, I may not completely agree with some of the conclusions and additional*

*experiments may help to strengthen the message proposed by the authors.*

*Major concerns*

*1. The authors only use a specific Mec1 background (mec1-100). Can we generalize their findings about this protective role of Rad9 to other Mec1-defective cells (either mec1D sml1D with very low HU doses or other hypomorphic mutants) or even to other checkpoint mutants (Mrc1D or Rad53 hypomophic for instance)?*

We now show that the synthetic effects on HU are not specific for a *mec1-100* background. In fact, the lack of Rad9 or the presence of Sgs1-G1298R exacerbated the HU sensitivity of cells carrying either *MEC1* deletion (kept viable by *SML1* deletion) (new Fig 2A) or the hypomorphic *mec1-14* allele (new Fig 2B). *RAD9* deletion also increased the HU sensitivity of cells carrying a Rad53 kinase defective variant (*rad53-K227A*) (new Fig 2C), whereas it had no effect on cells lacking the downstream kinase Chk1 (new Fig 2D).

*2. The authors propose that an increased resection by Dna2/Sgs1 (but not Exo1) at stalled forks is responsible for the poor HU survival and the replication deficiency of mec1-100 rad9D cells. Even if the mec-100 sgs1D double mutant already gives a strong HU sensitivity (Cobb et al., Genes Dev, 2005), it could be interesting to see whether sgs1 deficiency might somehow rescue the phenotype of mec1-100 rad9D cells. This would again argue that Dna2-Sgs1 dependent resection is toxic in mec1-100 rad9D cells.*

The double mutant *mec-100 sgs1Δ* shows a hypersensitivity to HU similar to that of *mec1-100 rad9Δ* cells. Therefore, it is not possible to test a possible suppression effect.

*3. The authors argue several times that "This Rad9 protective function is independent of checkpoint activation". However, rad9Δ mec1-100 cells are more sensitive to HU than sgs1-G1298R mec1-100 cells and display an undetectable Rad53 phosphorylation. The experiment with the rad9-STAA allele (defective in interacting with Dpb11) supports a checkpointindependent checkpoint of Rad9 since rad9-STAA mec1-100 cells are much more sensitive than mec1-100 cells while Rad53 activation is similar in both strains. However, at 3mM HU in Figure 4A, rad9D mec1-100 cells are more sensitive than rad9-STAA mec1-100, raising the possibility that the phenotypes observed in rad9D mec1-100 are partially dependent on a defect in checkpoint activation, in addition to the role of the Rad9-Dpb11 complex in antagonizing DNA resection. A role for Rad9 in promoting checkpoint signalling could also explain why the phenotypes of the rad9D mec1-100 are more dramatic than those of the sgs1-G1298R mec1-100 cells. Finally, the mec1-100 background already constitutes a particular checkpoint deficiency so the authors may want to be more precautious when stating that the Rad9 effect is checkpoint-independent.*

We agree with the reviewer and we have mentioned in the text that complete loss of checkpoint activation in *rad9*D *mec1-100* cells could explain the increased HU sensitivity of *rad9*D *mec1-100* compared to *rad9-STAA mec1-100* and *sgs1-G1298R mec1-100* cells (see pag 12). We have also removed “checkpoint independent” from the title of the paragraph.

*4. Related to point 3. Given the known anti-checkpoint role of Slx4 (Ohouo, Nature, 2013) in antagonizing Rad9-Dpb11 dependent signalling, a WB assessing Rad53 activation in the strains used in figure 4C could be informative. Indeed, Slx4 deficiency suppresses the HU sensitivity of mrc1D cells, likely by restoring Rad53 activation (Ohouo, Nature, 2013). It is conceivable that Slx4 deficiency similarly suppresses the HU sensitivity of mec1-100 cells by restoring a "WT" Rad53 activation.*

We have analyzed Rad53 phosphorylation in the strains used in Fig. 4C. Consistent with a requirement of Mec1 in restoring Rad53 phosphorylation, we found that deletion of *SLX4* in *mec1-100* cells did not increase Rad53 phosphorylation, indicating that suppression of the HU hypersensitivity of *mec1-00* cells is not due to restored Rad53 phosphorylation. The data are shown in the new Fig 6B.

*5. The authors propose that the "hyperstabilization of Rad9-Dpb11 interaction suppresses the*

 

*HU hypersensitivity of mec1-100 cells". This is based on the known competition between Slx4 and Rad9 for Dpb11 (Ohouo, Nature, 2013). However, I am not aware that such a competition exists between Fun30 and Rad9 for Dpb11 binding, which is presented as the rationale for the use of Fun30 and Slx4 mutants. Could the authors eventually show (by coIP) that the interaction between Rad9 and Dpb11 is actually increased in the absence of Slx4 (as expected) or in the absence of Fun30? On the contrary, is the interaction between Slx4 and Dpb11 increased in the rad9-STAA mec1-100 cell lines?*

We have repeated the coimmunoprecipitation experiment reported in Ohouo et al., 2013 showing that the Rad9-Dpb11 interaction is increased in MMS-treated *slx4*D cells. However, we did not detect any increase in Dpb11-Rad9 interaction either in MMS- or in HU-treated *slx4*D cells (and also in *fun30*D cells)(Figure for referees not shown). Because Slx4-Dpb11 interaction has been reported to be
promoted by Mec1-dependent Slx4 phosphorylation (Liu et al., 2017), the increased Dpb11-Rad9 interaction in *slx4*D cells reported in Ohouo et al., 2013 is not in agreement with the finding (by the same authors) that Rad9-Dpb11 interaction is not affected by the lack of Mec1 (Liu et al., 2017). Furthermore, while Slx4 binds to BRCT1 and 2 domains of Dpb11, Rad9 has been reported also to bind BRCT 3 and 4 (Gritenaite et al., 2014). Therefore, additional experiments are required to verify whether the functional antagonism between Rad9 and Slx4 can be explained simply because they compete for Dpb11 binding. As the molecular mechanism(s) by which Slx4 and Fun30 counteract the inhibitory action of Rad9 is not the focus of our manuscript, we decided not to include the coimmunoprecipitation experiments. We did not mention in the manuscript "hyperstabilization of Rad9-Dpb11 interaction".

*Moreover, the interaction between Slx4 and Dpb11 (but not between Dpb11 and Rad9) is strongly dependent on Mec1 after DNA damage (Liu, JCB, 2017). What about the HUinduced Slx4 phosphorylation in a mec1-100 background? Is it decreased thus weakening the Dpb11-Slx4 interaction? If yes, the Dpb11-Rad9 interaction might be stabilized in mec1-100 cells and represent a strong block to DNA resection, which might contribute to the dependency of mec1-100 cells on Rad9?*

It has been reported that Slx4 undergoes changes in its electrophoretic mobility due to phosphorylation events dependent on the Mec1 kinase, whereas HU treatment did not (Flott et al., 2007). We have repeated the experiment and we got similar results (Figure for referees not shown). Thus, we cannot use this assay to test whether the dependency of HU-treated *mec1-100* on Rad9 is due to reduced Slx4 phosphorylation. Furthermore, the lack of Rad9 exacerbated also the HU sensitivity of cells defective for the Rad53 checkpoint kinase (new Fig 2C), which is not involved in Slx4 phosphorylation (Flott et al., 2007). We have mentioned the different effect of MMS and HU treatment on Slx4 electrophoretic mobility in the text (see pag 12/13).

*6. I have several concerns about Figure 5 and the conclusions the authors draw from their data.*
*6a: Why the level of ssDNA at ARS607 (and at 0.5kb) does not decrease over time in WT cells, as would be expected from the Rpa1 ChIP data showing a decrease in Rpa1 binding over time?*
The pick of RPA association detected by ChIP 20 minutes after release in HU (new Fig 8A) of wild type cells correlates with that of DNA pole (new Fig 3E) and it likely represents ssDNA engaged by the replisome. ChIP analysis should detect Rpa1 bound at ssDNA generated both symmetrically and asymmetrically. By contrast, as the ssDNA molecules can re-anneal to each other upon DNA extraction and deproteinization, the signal we detected directly by qPCR in Figure 5 (now Figure 7) could represent preferentially ssDNA gaps generated asymmetrically (and therefore that cannot reanneal).
Therefore, it is possible that the ssDNA detected by qPCR does not decrease as it did Rpa1 association due to an underestimation of the pick of ssDNA generated symmetrically after 20 minutes after release. We have discussed this point in the result section (see pag 15).

*6b: Why is there no significant ssDNA generation in WT cells beyond 1,7kb from the ARS over time? I would expect that as replication proceeds away from the ARS, ssDNA generation would be detectable at later times points at these loci.*
As we analyzed a population of cells, and the amount of ssDNA we detected was low, it is possible

 

that replication forks lose their synchrony when they proceed away from the ARS and this does not allow to detect ssDNA generation at later time points.

*6c: How can the authors be sure than the ssDNA they detect originates from resection rather than uncoupling between the replicative helicase and the polymerase(s)?*
We tested whether the *dna2-1* allele decreases the amount of ssDNA in *mec1-100 sgs1-G1298R* and *mec1-100 rad9*D (see also point 6e). We found that *dna2-1 rad9*D *mec1-100* and *dna2-1 sgs1-G1298R mec1*-100 showed a decrease in ssDNA generation at stalled replication forks compared to *rad9*D *mec1-100* and *sgs1-G1298R mec1-100*, respectively. This finding is consistent with a model in which this ssDNA originates mainly from nucleolytic processing rather than from uncoupling events. These data have been added in new Figure 7.

*Do the authors think that this is due to the resection of the nascent strands and in this case, lagging and/or leading strand? Or could it be the consequence of a DSB at collapsed forks followed by a "classical" DNA end resection? Have the authors looked at whether there is an increase in DSBs in the double mutants? One source of collapsed forks could be the action of a structure-specific endonuclease (such as Mus81-Mms4) at unprotected stalled forks.*
We found that the lack of Mus81 does not suppress the HU hypersensitivity of *mec1-100 rad9*D *(*Figure EV1*)*, arguing against a role for Mus81 in generating DSBs in these cells. Thus, although we cannot exclude that other endonucleases can cleave DNA at stalled replication forks, the role of Dna2 in Okazaki fragment maturation prompts us to favor the hypothesis that Rad9 prevents the activity of Dna2 to degrade 5' ends generated at nascent lagging strands. We have discussed this point in the discussion section.

*6d: why does ssDNA accumulate over time both at the ARS (without reaching a plateau) and at distant loci (3kb and 5kb) in the double mutants rad9D mec1-100 and sgs1-G1298R mec1-100? Could this be actually explained by the specific and continuous resection of the Okasaki fragments on the lagging strand by Dna2/Sgs1 in the double mutants? But if this is the case, resection could seem slow?*
One possibility is that resection is slow. Alternatively, as we analyzed a population of cells, resection could start asynchronously from the replication origin, as it did at DNA double-strand breaks (Shroff et al., 2004). It is very difficult to discriminate between these two possibilities while testing a population of cells and not a single cell.

*6e: If this ssDNA increase is due to resection, one could expect that this could be reversed when combined with a defect in resection. Can the authors analyse ssDNA generation (eventually combined with Rpa1 ChIP analysis) in a dna2-1 mec1-100 rad9D background and see whether it is decreased compared to mec1-100 rad9D?*
See response to point 6c.

*6f: Is it possible to more directly address resection by BrdU ChIP to see whether neosynthetized DNA is actually resected over time?*
We detected a decrease in BrdU signal by ChIP in *mec1-100 rad9*D and *mec1-100 sgs1-G1298R* cells. However, as these mutants are defective in completing DNA replication, it not possible to conclude that the decrease is due to resection of newly synthesized strands rather than to a reduced BrdU incorporation. For this reason, these data have not been included in the manuscript.

*To conclude, I think this section needs clarification and that a model would help the readers to understand how resection happens in mec1-100 rad9D cells.*
We have discussed more deeply the results of Figure 5 (new Figure 7) both in the results and discussion sections and we have added a model in the new Figure 8D.

*Minor comments*
*1. Figure S1 should be incorporated in Figure 5.*
Figure S1 is now incorporated in the new Figure 8B.

*2. Visually, I would invert (upside down) the panel D of the Figure 2 with the high molecular weights DNA species at the top of the gel (and mentioning with an arrow these hMW DNA).*
We are sorry for the mistake. Now the gel has been flipped and an arrow has been added to indicate hMW DNA (new Fig 3D).

 

*Referee #2:*

*Rad9 and its human ortholog, 53BP1, have well documented roles in preventing extensive resection at DNA double-strand breaks (DSBs). However, the role of Rad9 is preventing resection at stalled replication forks has not previously been addressed. In this study, the authors present data supporting a role for Rad9 in protecting stalled forks from excessive degradation when the replication checkpoint is partially compromised. These studies make use of the mec1-100 hypomorphic allele, which is partially defective for the intra-S phase checkpoint and confers sensitivity to HU. The authors show that the sgs1-G1298R, a gain of function allele that is resistant to Rad9-mediated inhibition of resection at DSBs, synergizes with mec1-100, and this effect is also seen for the rad9 mutation. The most compelling data to support the proposal that Rad9 prevents extensive resection at stalled forks is presented in Fig 5, where the authors show increased ssDNA in the vicinity of ARS607 in checkpoint compromised cells defective for Rad9 or with the sgs1-G1298R mutation.*
*Given the current interest in replication fork stability the work it timely and suggests the role of Rad9 in suppressing resection is not limited to DSBs. There are some experimental concerns that need to be addressed:*

*The data presented in Fig 2D do not make sense. The nascent DNA labeled with BrdU should be chased to high molecular weight during the -HU recovery in the WT strain, instead it stays as low molecular weight intermediates. Is the gel flipped?*

We are sorry for the mistake. Now the gel has been flipped (new Fig 3D).

If the inviability of *mec1-100 sgs1-G1298R* cells is due to excessive resection, surely one would expect that reducing resection with the *dna2-1* mutation would rescue the *mec1-100 sgs1-G1298R* hypersensitivity to HU (Fig 3). Although *dna2-1* does partially suppress the hyper-HU sensitivity of the *mec1-100 rad9* mutant it should also suppress *mec1-100 sgs1-G1298R*. Given the complication of asking for survival as a read out and the potential of other effects of *exo1* and *dna2-1* on replication and recovery from arrest, I recommend the authors monitor ssDNA formation at ARS607 in *mec1-100 sgs1-G1298R exo1*, *mec1-100 sgs1-G1298R dna2-1* and *mec1-100 rad9 dna2-1* strains to ensure the increase in ssDNA is really due to resection by Dna2 nuclease. I suspect there could be some contribution from Exo1 in the *mec1-100 rad9* strain because Rad53 activation in response to HU is abolished in these cells and Exo1 phosphorylation by Rad53 negatively regulates its activity.

We monitored ssDNA formation in *mec1-100 sgs1-G1298R dna2-1* and *mec1-100 rad9*D *dna2-1* cells. We found that the presence of the *dna2-1* allele decreased the amount of ssDNA in both *rad9Δ mec1-100* and *sgs1-G1298R mec1-100* cells (new Fig 7), strongly suggesting that the ssDNA accumulated in the above double mutants is caused by an unregulated nucleolytic processing of stalled replication forks by Sgs1-Dna2. Regarding Exo1, this nuclease has been already reported to be involved in ssDNA generation in checkpoint mutants upon replication stress (Cotta-Ramusino et al., 2005; Segurado and Diffley, 2008). As our genetic tests did not support a role for Rad9 in limiting ssDNA generation by inhibiting Exo1 action at stalled replication forks, we have not tested the effect of *EXO1* deletion in *mec1-100 rad9* and *mec1-100 sgs1-G1298R* cells.

*The mec1-100 mutant has a delayed checkpoint response as measured by Rad53 activation. Is the amount of Rad9 bound to stalled forks when cells are treated with HU decreased in the mec1-100 and mec1-100 sgs1-G1298R mutants? Rad9 binding could be assessed by ChIP at ARS607.*

Mec1-100 cells show a defect not only in Rad53 but also in Ddc2 phosphorylation upon DNA replication stress (Paciotti et al., 1998). As Ddc2 phophorylation depends on Mec1 and not on Rad9, the checkpoint defect of *mec1-100* cells is likely due to defective Mec1-dependent signaling rather than from defective Rad9 association to DNA ends. Consistent with this hypothesis, *mec1-100* cells do not show increased ssDNA generation, suggesting that Rad9 still exerts its protective function in these cells. In any case, we measured Rad9 association by ChIP at ARS607 and we found that Rad9 is associated at ARS607 in both wild type and *mec1-100* cells, with *mec1-100* showing a slight increased Rad9 persistence compared to wild type cells. This data has been added in new Figure 5A.

 

*The authors use the mec1-100 allele to partially compromise the replication checkpoint. Is fork degradation seen in mec1 null or chk1 mutants?*

We now show that the lack of Rad9 or the presence of Sgs1-G1298R exacerbated the HU sensitivity of cells carrying either *MEC1* deletion (kept viable by *SML1* deletion) (Fig 2A) or the hypomorphic *mec1-14* allele (new Fig 2B). *RAD9* deletion also increased the HU sensitivity of cells carrying a Rad53 kinase defective variant (*rad53-K227A*) (new Fig 2C), whereas it had no effect on cells lacking the downstream kinase Chk1 (new Fig 2D).

*Does the increase in ssDNA at replication forks in mec1-100 sgs1-G1298R or mec1-100 rad9 cells cause more fork collapse?*

To measure fork collapse, we tested the association of DNA pole at the replication forks by ChIP, comparing wild-type and mutant strains synchronously entering S phase in the presence of HU. We found that pole was efficiently bound with ARS607 and ARS305 within 20 minutes after release in HU in wild type cells (new Fig 3E). By contrast, we could detect a diminished DNA pole association in *mec1-100* cells compared to wild type cells and a further decrease of this association in both *sgs1-G1298R mec1-100* and *rad9Δ mec1-100* (new Fig 3E).

*Minor comments*
*P. 6: Sgs1 is not the only yeast RecQ helicase, Hrq1 is also in the RecQ family.*
We have modified the sentence.

*Fig 4C: The spot assays are messy; are the large colonies on 20 and 25 mM HU plates contaminants or suppressor mutants?*
The large colonies are suppressors. In any case, we have repeated the experiments and we now show plates in which spontaneous suppressors are less apparent.

*Referee #3:*

*The manuscript by Villa and colleagues describes a detailed analysis of the protection of compromised replication forks by a checkpoint independent function of Rad9/53BP1. They present arguments indicating that Rad9 protective function act through the inhibition of the Sgs1/Dna2 mediated resection independently of its checkpoint function. They also provide genetic arguments suggesting that Rad9 is recruited by Dpb11 at compromised replication forks.*
*In general, this work is of high quality, the manuscript is well written and this paper addresses mechanistic questions that are of great interest to researchers in the replication field.*
*The results presented are based on the analysis of a combination of genetic interaction with previously characterized mutants. However most of the mutants used haven't been previously characterized for their behavior in response to HU and this should be systemically done in this paper. I thus consider that some controls (listed below) are lacking at present.*

*Major comments:*

*- Figure 1F: The double rad9 sgs1-G1298R is missing.*
The double mutant *rad9Δ sgs1-G1298R* is now shown in Figure 1F.

*- Figure 2B: The single rad9, sgs1-G1298R and the double rad9 sgs1-G1298R should be shown.*
The *rad9Δ* and *sgs1-G1298R* single mutants, as well as *rad9Δ sgs1-G1298R* double mutants, are now shown in Figure 3B.

*- Figure 2D: The high molecular weight products expected in WT strains appear at the bottom of the blot. Isn't this blot flipped upside down?*
We are sorry for the mistake. Now the blot has been flipped (new Fig 3D).

*- Figure 3A: The exo1 sgs1-G1298R control is missing.*
The double mutant *exo1Δ sgs1-G1298R* is now shown in the new Figure 4A.

 

*- I also have a concern on the conclusion drawn from panel 3 B and C. Although I agree that the lack of Rad9 requires Dna2 to exacerbate the HU sensitivity of the mec1-100 mutant, I don't think that the authors can say the same for the expression of Sgs1-G1298R. Indeed, they observed that the sgs1- G1298R mec1-100 mutant is less sensitive than the sgs1-G1298R dna2-1 mec1-100 and as sensitive than the dna2-1 mec1-100. This, I think only suggests that Sgs1-G1298R and Dna2-1 act in the same pathway and that the dna2-1 mutant is either more severely affected or has an additional function.*
We have modified the text according to the reviewer's concern.

*- Figure 4B: Rad53 phosphorylation should also be tested in the rad9-Y798A and rad9-STAA single mutants.*
We now show Rad53 phosphorylation in *rad9-Y798A* and *rad9-STAA* single mutants in the new Figure 5C.

*- I could also be worth testing the whole set of single and double mutants for their progression through S phase upon HU treatment and see whether Δfun30 and Δslx4 restores or not the Rad53 phosphorylation in mec1-100 mutants upon HU.*
We have analyzed Rad53 phosphorylation in *mec1-100 slx4Δ* and *mec1-100 fun30Δ* cells compared to each single mutant. We found that deletion of *SLX4* did not increase Rad53 phosphorylation in *mec1-100* cells, indicating that suppression of the HU hypersensitivity of *mec1-00* cells is not due to restored Rad53 phosphorylation. These data are shown in the new Figure 6B.
*Minor comments:*

*Sgs1 has a broad range of contributions at replication forks, which often renders it difficult to determine the exact interplay of the partially redundant pathways, that enable fork restart, checkpoint activation. Notably in this manuscript, understanding why both deletion of SGS1 and a uncontrolled resection by the sgs1-G1298R mutant both lead to increased HU sensitivity in response to HU in mec1-100 mutant possibly requires a background on Sgs1 function at replication forks. I thus suggest the authors to add a paragraph on this in the introduction to help non-specialized readers.*
*For the same reason, the rational of testing mus81 mutants and an interpretation on the absence of synergism in the mus81 sgs1-G1298R should be given p6.*

We have now added in the introduction a paragraph on the role of Sgs1 in DNA replication, as well as the rationale of testing *mus81Δ*.

*I would like to emphasize the clarity of this manuscript and the high quality of the data presented. I will be happy to see it published soon.*

2nd Editorial Decision 27 November 2017

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it. I am happy to tell you that all referees support its publication now. Referee 1 only has one minor suggestion that I would like you to address before we can proceed with the official acceptance of your manuscript.

Please also correct the reference style, the EMBO reports style can be found in EndNote. Not more than 10 authors may be listed before et al.

Please send us a short summary of the findings and their significance, bullet points highlighting key results and a synopsis image of 550 pixels wide x 200-400 pixels high. You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

 

Referee #1:

The authors have done an impressive work to improve the quality of their manuscript.
They nicely addressed to me all the points raised in the first round of revision, adding new experimental data and more discussion.

This article will be of high interest in the field, especially in light of the very recent finding showing that the Rad9 human ortholog 53BP1 plays a role in replication fork restart (Xu, Elife, 2017). This role is still elusive and this paper showing that Rad9 can counteract DNA degradation at stalled replication forks in yeast will probably inspire future studies in mammalian cells.

I am enthusiastic to see it published soon in EMBO Reports.

I just spotted this on page 17:

The HU sensitivity of mec1-100 cells was only slightly increased by expression of either the rad9-Y798A or the hta1-S129A allele (Fig 5B), which abolishes γH2A generation and Rad9 association to H3-K79me, respectively
SHOULD BE
The HU sensitivity of mec1-100 cells was only slightly increased by expression of either the rad9-Y798A or the hta1-S129A allele (Fig 5B), which abolishes Rad9 association to H3-K79me and γH2A generation, respectively

Referee #2:

The authors have adequately addressed all of my comments.

Referee #3:

The authors have carefully responded to all the reviewers' comments with a series of convincing additional experiments. All of my concerns have been addressed and I therefore recommend publication.

2nd Revision - authors' response 29 November 2017

We have corrected the mistake in the sentence mentioned by reviewer 1, as well as the reference style. Please find enclosed the revised version of the manuscript, together with a short summary, bullet points and a synopsis image

 

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

| |
|---|
| Corresponding Author Name: Maria Pia Longhese |
| Journal Submitted to: EMBO Reports |
| Manuscript Number: EMBOR-2017-44910V2 |

**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.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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.**

## B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | The sample size was chosen based on our experience. The fact that we observed a significant difference means that the power of the test (and therefore sample size) was sufficient to detect the effect. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | NA |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | We did not exclude any sample |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | No, it was not pertinent to our experiments |
| For animal studies, include a statement about randomization even if no randomization was used. | NA |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | No. Blinding of the investigator is not requested in these kind of experiments |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | NA |
| 5. For every figure, are statistical tests justified as appropriate? | Yes, the statistical test used (t-test) is reported in every figure legends. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | The data meet the assumption of the test. This was assessed by using test KS (Kolmogorov Smirnov) |
| Is there an estimate of variation within each group of data? | yes, standard deviation |

## USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

| | |
|---|---|
| Is the variance similar between the groups that are being statistically compared? | Variances between groups are in general similar and we adjusted the t-test whenever variance was different. |

## C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | The source of the antibodies is described in the Materials and methods section: pag. 20 for anti-Rad53 antibodies, pag 21 for anti-Flag, anti-Rad51 and anti-Myc antibodies and pag 22 for anti-Brdu antibodies. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | NA |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | NA |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | NA |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | NA |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | NA |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | NA |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

## F- Data Accessibility

| | |
|---|---|
| 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'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | NA |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | NA |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

## G- Dual use research of concern

| | |
|---|---|
| 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 |