EMBO reports

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Escape of Sgs1 from Rad9 inhibition reduces the requirement for Sae2 and functional MRX in DNA end resection

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(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.)

Editor: Esther Schnapp

1st Editorial Decision 12 November 2014

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, all referees agree that the findings are potentially interesting and the data convincing. However, they also have a few suggestions for how the study could be further improved. Given the rather few suggestions, I think that all of them should be addressed, except for point 1 of referee 1, as both referees 2 and 3 indicate in their cross-comments that a further characterization of the sgs1-ss mutant - while certainly very interesting - goes beyond the scope of this study. However, referee 3 also remarks that the last question in point 1, whether the sgs1-ss mutation removes MRX from DNA ends, could be addressed, as this seems to be feasible.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. 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.

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:

Manuscript reports identification and characterization of sgs1-ss mutant that suppresses drug sensitivity of sae2 deletion by allowing the cells to overcome inhibition of Rad9 on resection and thereby improving resection at the early resection stage. As always, Dr. Longhese's work and the results are clear-cut and well presented to be highly convincing. Nevertheless, the story appears to have two weaknesses that can be corrected rather easily by few additional experiments; one is that the manuscript did not tell you much about what truly sgs1-ss is defective and how it could allow Dna2 to bypass inhibitory actions of rad9. Does it have more efficient helicase activity? What other functions of sgs1-ss are also affected by this mutation? Does it do better at HJ dissolution? Could the mutation remove MRX from DNA ends as the author showed previously in sae2 deleted cells?

The second one is that the story is too much reliant on resection measurement alone but not the product formation that could also be a nice assay to confirm the validity of the conclusion. Can sgs1-ss improve the SSA product formation in sae2 deletion or even in wild type SAE2+ cells? Could ku sgs1-ss cells do super-fast resection and SSA product formation? Does it also affect telomere resection as shown by Lydall group in cdc13-1 cells? Could exo1 sae2 sgs1-ss form SSA products?

Many of these can be addressed very easily as the authors used all these assays before and have strong expertise in all of these assays. The outcomes will help back-up the authors' argument further. As it currently stands, one may wonder if the effect of sgs1-ss is more indirect such as it affects checkpoint (checkpoint looks weak based on rad53 phosphorylation) than specific.

Minor point: Fig. 5C, the red color is also rad9 results...

I also suggest to include ChIP assay results at regions further away from the break such as 5-7 kb where extensive resection could be monitored.

Referee #2:

Bonetti et al. Embo reports.

To repair double-strand DNA breaks by homologous recombination, the 5'-terminated DNA strand must first be resected. This process is typically initiated by the Sae2 and Mre11-Rad50-Xrs2 proteins, which is followed by either of two long-range pathways, dependent on either Sgs1-Dna2 or Exo1. The activity of these pathways must be controlled and regulated. The manuscript by Bonetti et al. focuses on one of these negative regulators, the Rad9 protein. Specifically, the authors started by looking for suppressors of CPT sensitivity of sae2-delta strains. They identified a point mutation in the Sgs1 helicase (Sgs1-ss). Subsequent work revealed that Sgs1-ss is refractory to inhibition by Rad9, by having likely an increased affinity to bind and/or persist at DNA ends. Thus, Sae2 helps overcome resection inhibition by Rad9. Overall, the manuscript is well written, covers an important topic, and the experiments are of a very good quality. I only have minor points.

1. Can authors describe in more detail how did they verify that the suppressor phenotype is indeed specific (dependent on) the point mutation within Sgs1? It would be helpful to perform a sgs1-delta complementation experiment with either wt of Sgs1-ss mutant expressed from a centromeric plasmid.

2. This is more a discussion point. The authors state that Sgs1-Dna2 can substitute for Mre11 nuclease in the absence of Rad9. This is based on the observation that Sae2 helps overcome the Rad9 inhibition even when nuclease activity of Mre11 is eliminated (but not in the absence of Mre11). I think one has to be careful with this interpretation. The drug that is mostly used in this study is CPT - while according to some reports this causes protein-DNA adducts at the ends of DSBs, this is likely dependent on the effective concentration of CPT. It was shown by the Lopes laboratory (Ray Chaudhuri et al, NSMB, 2012) that covalent topoisomerase adducts are not necessarily the result of CPT treatment (at least in mammals). Furthermore, there may be other activities that can substitute the Mre11 nuclease in case of covalent topo adducts. I doubt that Sgs1-Dna2 could substitute for Mre11 nuclease when it is known to be absolutely required (e.g. in Spo11 removal). I am not asking the authors to do that experiment (would be above the scope of this paper), but rather illustrate the point that overall generalization may be misleading. I think the results show that the Sgs1-ss mutation does not bypass the requirement for Sae2 to activate the Mre11 nuclease. Rather, since the rescue is independent of the Mre11 nuclease, the Sgs1-ss mutation renders the system refractory to Rad9 inhibition.

Once again, I enjoyed reading this manuscript and will be happy to see it published soon.

Referee #3:

Bonetti et al. identified a novel Sgs1-ss mutant that suppresses the CPT and MMS hypersensitivity of a sae2Δ strain most likely by counter acting the inhibitory role of Rad9 at DSBs. This finding is relevant for the recombination field as it clarifies the control of DNA end resection, the first step engaging recombination.

Overall the data are clear and well presented. I have minor comments on the manuscript itself, and one major comment that I would like to be addressed.

Major comment
In a recent previous paper in EMBO reports (Marina et al 2014), the Longhese group reported the influence of Rif1Δ on DNA end resection. They notably showed that in the absence of Rif1, there is an increase in Rad9 binding at DSBs but no change in Sgs1 or Exo1 binding. Here, they see that the absence of Rad9 leads to an increase in Sgs1 binding at DSBs. It looks like there is a discrepancy between these two observations that the author should address.

Minor comment
- Recent findings point out the importance of chromatin in controlling resection, and especially extensive resection, since the lack of chromatin remodelers impair resection to the same extent as the lack of resection players. This concept could be added to the second paragraph of the introduction.

- The screening strategy is extremely interesting. Without diluting the message of the paper, a more exhaustive description of it could be could benefit the entire community. One may have expected Top1 mutants to be among the best suppressors of sae2Δ hypersensitivity.

- p6, description of Fig 1E: need to include in the text that the cells used contain a galactose inducible DSB site to follow the rationale of the experiment.

- p8: the references go from 22 to 28, I guess they should be reformatted.

- Fig 3A: Because of the recent publication of the Longhese group on Rif1 controlling resection in G1, I think showing the resection level in G1 phase in rif1Δ cells, rif1Δ rad9Δ cells and rif1Δ SGS1-ss cells would be very helpful for the community. Notably, it would show whether the Sgs1-ss mutant could overcome the Rad9 barrier in G1 or not.

- p9: "DSB resection in the absence of Rad9 is more dependent on Sgs1 than on Exo1 »:
The interpretation of the authors is misleading. The data say that hyper-resection in rad9Δ cells depends on Sgs1, but Exo1 mediated DSB resection allows wild type levels of resection in the absence of Rad9 (Fig4 A and B).

- Figure 4. The authors should provide the ssDNA accumulation profile of the double mutant SGS1-ss exo1: this would allow to compare the resection level between the SGS1-ss exo1Δ mutant and the rad9Δ exo1Δ mutants and determine to which extent the Sgs1-ss protein bypasses the presence of Rad9.

- Fig5 C and D:
color code problem. I guess the red bars should be green?...
it would be interesting to see the Exo1 ChIP data in the absence of Rad9 to determine if the increase in ChIP signal at DSBs is specific to Sgs1 or not in the absence of Rad9.

1st Revision - authors' response 12 December 2014

Response to reviewers

Referee #1:

Manuscript reports identification and characterization of sgs1-ss mutant that suppresses drug sensitivity of sae2 deletion by allowing the cells to overcome inhibition of Rad9 on resection and thereby improving resection at the early resection stage. As always, Dr. Longhese's work and the results are clear-cut and well presented to be highly convincing. Nevertheless, the story appears to have two weaknesses that can be corrected rather easily by few additional experiments; one is that the manuscript did not tell you much about what truly sgs1-ss is defective and how it could allow Dna2 to bypass inhibitory actions of rad9. Does it have more efficient helicase activity? What other functions of sgs1-ss are also affected by this mutation? Does it do better at HJ dissolution?

These are certainly interesting points. As the editor pointed out that additional insights into how Sgs1-ss promotes DNA end resection are not required for publication of the manuscript in EMBO reports, these aspects will be investigated in future studies.

Could the mutation remove MRX from DNA ends as the author showed previously in sae2 deleted cells?

We now show that Sgs1-ss suppresses the persistence of the MRX complex at the DSB ends in *sae2Δ* cells (Figure 2C).

The second one is that the story is too much reliant on resection measurement alone but not the product formation that could also be a nice assay to confirm the validity of the conclusion. Can sgs1-ss improve the SSA product formation in sae2 deletion or even in wild type SAE2+ cells?

We now show that Sgs1-ss accelerates DSB repair by SSA in a wild type context and suppresses the SSA defect of *sae2Δ* cells (Figure 3D and E).

Could ku sgs1-ss cells do super-fast resection and SSA product formation?

Since the Ku complex prevents DSB resection specifically in G1, we investigated whether Sgs1-ss enhances resection in G1-arrested *kuΔ* cells. We found that Sgs1-ss is not capable to allow DSB resection in G1 either in the presence or in the absence of Ku (Figure 4D and E, Supplementary Figure S2). As Sgs1-ss functions in DSB resection depends on Dna2, whose activity requires Cdk1-mediated phosphorylation (Chen et al., 2011), the inability of Sgs1-ss to allow DSB resection in G1 may be due to the requirement of Cdk1 activity to support Dna2 and therefore Sgs1-ss function in DSB resection. Given that Sgs1-ss does not allow DSB resection in G1, we have not monitored SSA in *SGS1-ss ku70Δ* cells.

Does it also affect telomere resection as shown by Lydall group in cdc13-1 cells?

In contrast to deletion of *EXO1*, deletion of *SGS1* has been reported to enhance the temperature sensitivity of *cdc13-1* cells (Ngo and Lydall, 2010), suggesting that Sgs1 contributes to the stability of uncapped telomeres in *cdc13-1* mutant through an unknown mechanism. Therefore, although the investigation of the effect of Sgs1-ss on telomere resection in *cdc13-1* mutant is interesting, it is above the scope of this manuscript as in this case it could not be used as readout for what happens at DNA DSBs.

Could exo1 sae2 sgs1-ss form SSA products?
We now show that Sgs1-ss suppresses the DSB resection defect of *exo1Δ* cells (Supplementary Fig S3). We have not tested SSA because *EXO1* deletion affects only long range resection and therefore it impairs very slightly DSB repair by SSA in YMV45 strain, in which the homologous sequences are 4.5 kb apart. Deletion of *EXO1* affects SSA in YMV80 strain, in which the homologous *LEU2* sequences are 25 kb apart. However, the possible results with this strain are biased by the fact that the DNA intervening sequence between the two tandem *LEU2* repeats contains a DNA sequence that forms a hairpin structure that need to be resolved by MRX-Sae2.

Many of these can be addressed very easily as the authors used all these assays before and have strong expertise in all of these assays. The outcomes will help back-up the authors' argument further. As it currently stands, one may wonder if the effect of sgs1-ss is more indirect such as it affects checkpoint (checkpoint looks weak based on rad53 phosphorylation) than specific.
We agree with the reviewer. However, it is unlikely that Sgs1-ss suppression of *sae2Δ* hypersensitivity to DNA damaging agents is due to an effect on checkpoint activation, because generation of the HO-induced DSB leads to similar amounts of phosphorylated Rad53 in *sae2Δ* and *sae2Δ SGS1-ss* cells within the first 12 hours (Figure 2B).

Minor point: Fig. 5C, the red color is also rad9 results...
We have corrected the mistake.

I also suggest to include ChIP assay results at regions further away from the break such as 5-7 kb where extensive resection could be monitored.
We now show Sgs1 and Sgs1-ss recruitment at 5.4 kb from the DSB (Figure 6C).

Referee #2:
To repair double-strand DNA breaks by homologous recombination, the 5'-terminated DNA strand must first be resected. This process is typically initiated by the Sae2 and Mre11-Rad50-Xrs2 proteins, which is followed by either of two long-range pathways, dependent on either Sgs1-Dna2 or Exo1. The activity of these pathways must be controlled and regulated. The manuscript by Bonetti et al. focuses on one of these negative regulators, the Rad9 protein. Specifically, the authors started by looking for suppressors of CPT sensitivity of sae2-delta strains. They identified a point mutation in the Sgs1 helicase (Sgs1-ss). Subsequent work revealed that Sgs1-ss is refractory to inhibition by Rad9, by having likely an increased affinity to bind and/or persist at DNA ends. Thus, Sae2 helps overcome resection inhibition by Rad9. Overall, the manuscript is well written, covers an important topic, and the experiments are of a very good quality. I only have minor points.

1. Can authors describe in more detail how did they verify that the suppressor phenotype is indeed specific (dependent on) the point mutation within Sgs1? It would be helpful to perform a sgs1-delta complementation experiment with either wt of Sgs1-ss mutant expressed from a centromeric plasmid.
We have now described in more details the screening and how we have determined that the *SGS1-ss* mutation was responsible for the suppression in the Methods section.

2. This is more a discussion point. The authors state that Sgs1-Dna2 can substitute for Mre11 nuclease in the absence of Rad9. This is based on the observation that Sae2 helps overcome the Rad9 inhibition even when nuclease activity of Mre11 is eliminated (but not in the absence of Mre11). I think one has to be careful with this interpretation. The drug that is mostly used in this study is CPT - while according to some reports this causes protein-DNA adducts at the ends of

DSBs, this is likely dependent on the effective concentration of CPT. It was shown by the Lopes laboratory (Ray Chaudhuri et al, NSMB, 2012) that covalent topoisomerase adducts are not necessarily the result of CPT treatment (at least in mammals). Furthermore, there may be other activities that can substitute the Mre11 nuclease in case of covalent topo adducts. I doubt that Sgs1-Dna2 could substitute for Mre11 nuclease when it is known to be absolutely required (e.g. in Spo11 removal). I am not asking the authors to do that experiment (would be above the scope of this paper), but rather illustrate the point that overall generalization may be misleading. I think the results show that the Sgs1-ss mutation does not bypass the requirement for Sae2 to activate the Mre11 nuclease. Rather, since the rescue is independent of the Mre11 nuclease, the Sgs1-ss mutation renders the system refractory to Rad9 inhibition.
Since this point is very important, we have investigated whether Sgs1-ss could bypass Sae2 requirement in resecting meiotic DSBs, where the Sae2/MRX-mediated endonucleolytic cleavage is absolutely required to initiate DSB resection. We found that meiotic DSBs generated at the *THR4* hotspot disappeared in both wild type and *SGS1-ss/SGS1-ss* cells about 4 hours after transfer to sporulation medium, while they persisted until the end of the experiment in both *sae2Δ/sae2Δ* and *sae2Δ/sae2Δ SGS1-ss/SGS1-ss* diploid cells. Thus, Sgs1-ss-Dna2 cannot substitute the endonucleolytic clipping by Sae2/MRX when this is absolutely required to initiate DSB resection.
These data are reported in the Supplementary Figure S1.

Once again, I enjoyed reading this manuscript and will be happy to see it published soon.

Referee #3:
Bonetti et al. identified a novel Sgs1-ss mutant that suppresses the CPT and MMS hypersensitivity of a sae2Δ strain most likely by counter acting the inhibitory role of Rad9 at DSBs. This finding is relevant for the recombination field as it clarifies the control of DNA end resection, the first step engaging recombination. Overall the data are clear and well presented. I have minor comments on the manuscript itself, and one major comment that I would like to be addressed.

Major comment
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As pointed out by the reviewer, we showed an increase of Rad9 binding, but not a decrease in Sgs1 binding, at the DSB in exponentially growing *rif1Δ* cells compared to wild type. However, the lack of *RIF1* by itself impairs DSB resection only in G1, whereas Rif1-mediated regulation of DSB resection in G2 and in exponentially growing cells was partially redundant with other resection activities (we reported a DSB resection defect only in *rif1Δ sae2Δ* and *rif1Δ exo1Δ* double mutants). Therefore, although Rad9 binding is higher in exponentially growing *rif1Δ* than in wild type cells, this increased binding is not enough to inhibit Sgs1 recruitment at the DSB and therefore DSB resection. One possibility is that the Rad9-mediated suppression of Sgs1 activity cannot be further improved by an increase of Rad9 recruitment at the DSB, but it can be impaired when Rad9 is absent or its function is bypassed by Sgs1-ss.
In the Rif1 paper, Sgs1 recruitment was shown only in exponentially growing cells and not in G1-arrested cells, because in G1 (where the lack of Rif1 impairs DSB resection in a Rad9-dependent manner) we could detect very low amount of Sgs1 bound at the DSB even in wild type cells (see also figure 6D of this manuscript). Therefore, it was not possible to determine whether the increase of Rad9 binding in G1-arrested *rif1Δ* cells lead to a reduction of Sgs1 recruitment. In summary, we do not see discrepancy between this manuscript and the Rif1 paper, because we cannot actually determine whether the increase of Rad9 binding that we observed in *rif1* deleted cells leads to a reduction in Sgs1 binding at the DSB in G1.

Minor comment
- Recent findings point out the importance of chromatin in controlling resection, and especially extensive resection, since the lack of chromatin remodelers impair resection to the same extent as the lack of resection players. This concept could be added to the second paragraph of the

introduction.
We have included chromatin remodelling proteins in the Introduction.

- The screening strategy is extremely interesting. Without diluting the message of the paper, a more exhaustive description of it could be could benefit the entire community. One may have expected Top1 mutants to be among the best suppressors of sae2Δ hypersensitivity.
We have included data regarding the mutations of the four additional clones we sequenced.

- p6, description of Fig 1E: need to include in the text that the cells used contain a galactose inducible DSB site to follow the rationale of the experiment.
We have described the strain in the text.

- p8: the references go from 22 to 28, I guess they should be reformatted.
We have corrected the mistake.

- Fig 3A: Because of the recent publication of the Longhese group on Rif1 controlling resection in G1, I think showing the resection level in G1 phase in rif1Δ cells, rif1Δ rad9Δ cells and rif1Δ SGS1-ss cells would be very helpful for the community. Notably, it would show whether the Sgs1-ss mutant could overcome the Rad9 barrier in G1 or not.
RAD9 deletion does not allow DSB resection in G1, but it enhances resection in G1-arrested *kuΔ* cells (Trovesi et al., 2011), indicating that Rad9 inhibits DSB resection in G1, but this function becomes apparent only when Ku is absent. To investigate whether Sgs1-ss was capable to counteract the inhibitory function of Rad9 in G1, we monitored DSB resection in *SGS1-ss* and *SGS1-ss ku70Δ* cells that were kept arrested in G1 by α-factor during HO induction. We found that Sgs1-ss is not capable to allow DSB resection in G1 either in the presence or in the absence of Ku (Figure 4D and E, Supplementary Figure S2). As Sgs1-ss functions in DSB resection depends on Dna2, whose activity requires Cdk1-mediated phosphorylation (Chen et al., 2011), the inability of Sgs1-ss to overcome Rad9-mediated inhibition in G1 may be due to the requirement of Cdk1 activity to support Dna2 and therefore Sgs1-ss function in DSB resection. Therefore, it is unlikely that Sgs1-ss suppresses the resection defect of *rif1Δ* cells in G1.

- p9: "DSB resection in the absence of Rad9 is more dependent on Sgs1 than on Exo1 »: The interpretation of the authors is misleading. The data say that hyper-resection in rad9Δ cells depends on Sgs1, but Exo1 mediated DSB resection allows wild type levels of resection in the absence of Rad9 (Fig4 A and B).
We agree with the reviewer and we have modified the sentence as suggested.

- Figure 4. The authors should provide the ssDNA accumulation profile of the double mutant SGS1-ss exo1: this would allow to compare the resection level between the SGS1-ss exo1Δ mutant and the rad9Δ exo1Δ mutants and determine to which extent the Sgs1-ss protein bypasses the presence of Rad9.
We show that DSB resection in *SGS1-ss exo1Δ* is similar to that of *rad9Δ exo1Δ* cells (Supplementary Figure S3).

- Fig5 C and D:
 # color code problem. I guess the red bars should be green?
We have corrected the mistake.

 # it would be interesting to see the Exo1 ChIP data in the absence of Rad9 to determine if the increase in ChIP signal at DSBs is specific to Sgs1 or not in the absence of Rad9.
We now show that the lack of Rad9 also increases the recruitment of Exo1 at the HO-induced DSB (Fig 6E). This finding is consistent with the observation that although the accelerated resection in *rad9Δ* cells mainly depends on Sgs1, also Exo1 contributes to resect DNA in *rad9Δ* cells.

2nd Editorial Decision 19 December 2014

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the comments from 2 referees, who both support publication of the study in our journal. Only a few minor changes will be needed before we can proceed with the official acceptance of your manuscript.

Can you please include the suggestion from referee 1 in the manuscript text?

I further noticed that section B in the author checklist has not been filled out. Given that you perform statistical analyses, all the questions under B. Statistics need to be answered (except for the questions on animal research). Please send us a new, completed checklist.

The figure legend for figure panel 2A does not mention "n". Has the experiment only been performed once, or is it the sum of several experiments? Please mention n in the legend.

I also would like to suggest to modify the before-last sentence in your abstract to:

" When inhibition by Rad9 is abolished by the Sgs1-ss mutant or by deletion of RAD9, the requirement for Sae2 and functional MRX in DSB resection is reduced. "

Please let me know whether you agree with these changes.

Today is my last day in the office, and I will only be back in January. You therefore have time until the 4th of January to send us the final files.

REFEREE REPORTS:

Referee #1:

The revision addressed most of my comments and those of other referee, is greatly improved at most of areas. Even if it is not essential, it would be helpful to discuss and compare their mutant with sgs1-D664A described by Rothstein Group as both showed genetic interaction with Sae2 and affect resection but by opposite manner.

Referee #2:

I believe that the additional experiments carried out in response to reviewers' comments strengthened the manuscript and I fully support its publication.

2nd Revision - authors' response 23 December 2014

We thank you for considering for publication in *EMBO Reports* a revised version of our manuscript “Escape of Sgs1 from Rad9 inhibition reduces the requirement of Sae2 and functional MRX in resection of DNA ends” by Diego Bonetti, Matteo Villa, Elisa Gobbini, Corinne Cassani, Giulia Tedeschi and Maria Pia Longhese.

We have modified the manuscript as follows:
-We have modified the sentence in the abstract.
-We have included in the text the suggestion from referee 1.
-We have added the “n” in the legend of Figure 2A
-We have filled-in the author checklist

Please find enclosed the final version of the manuscript.

We thank again you and the reviewers and we look forward to hearing from you.

3rd Editorial Decision 05 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

