

Competing interaction partners modulate the activity of Sgs1 helicase during DNA end resection

Kristina Kasaciunaite, Fergus Fettes, Maryna Levikova, Peter Daldrop, Roopesh Anand, Petr Cejka and Ralf Seidel.

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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

12th February 2019

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you can see, the reviewers expressed an overall interest in the study, but also raised some points that should be addressed in a revised version of the manuscript. Both reviewer 1 and 2 point out that the proposed regulation of Sgs1 by RPA binding should be experimentally demonstrated, and suggest using an RPA binding-deficient Sgs1 mutant to do so. In addition, both reviewers note that some experimental results are discussed without all data being shown in the manuscript or the extended files.

Should you be able to address these major concerns, as well as the various more specific technical and presentational issues raised by all three referees, then we would be happy to consider this study further for publication. I would therefore like to invite you to prepare and submit a revised manuscript.

REFeree REPORTS

Referee #1:

Kasaciunaite et al. attempt to understanding exactly how Sgs1 and Dna2 work together, and how RFA contributes to their unwinding function, using purely in vitro single molecule analysis. They also address in one figure, Top3/Rmi1 contributions to unwinding in vitro. In general, the paper is not very accessible to the non-single molecule expert and there seems to be a lot of "interpretation" based on the stretching of DNA, that this reviewer finds difficult fully accept. Either they need to significantly improve the description of the physical nature of the "elongated DNA molecule" or else

make it clear on what basis they make their interpretation of the traces presented as being "unwinding" or "re-zipping". There could be other reasons why a DNA molecule becomes longer (e.g. resolution of foldback structures, nicking ?).

Apart from data presentation, the English needs revision, and there are a few major experiments omitted. Many crucial controls/results are mentioned but not shown. Finally, with the addition of experiments using specific mutants (listed below) they can render the paper both more convincing and more biologically relevant. Due to these oversights and omissions, it is impossible at present to recommend acceptance, but the topic and insights are definitely interesting. Better controls, use of mutants and better explanation of their interpretations would make their results more appropriate for the EMBO J audience. These points are enumerated below.

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-P5, Consistently, no DNA length.....when omitting ATP or protein in the reaction (data not shown).

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3. They need to test the DNA duplex processing with Sgs1-r1 mutant protein (ref 25, Hegnauer et al., EMBO J, 2012), which lacks a major RPA binding domain. This will confirm the statement that RPA modulates SGS1 activity through protein interaction, not simply as roadblock. Testing this mutant would also allow one to evaluate the in vivo impact of their in vitro studies, because this mutant was extensively studied in vivo.

4. Is it possible to test the dna2 nuclease and helicase double mutant combined to address contribution of each function ? Is the effect the same or additive?

General comment: The slow rewinding of the DNA in presence of Sgs1 is interpreted as strand switching but the possibility of backtracking or dissociation and re-association on the same strand is never mentioned. Has this possibility already been excluded by previous research, or is it common knowledge that it is impossible ?

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Minor points:

Why is it stated that the histograms are presented {plus minus} SEM ? (Except Fig 5)

English needs help here: "however the underlying interplay of this interaction remains undefined."

In introduction

"mandate cognate RPA" (introduction page 4) Please improve English.

Intro last para: (A1)though functional synergies...., however, how Sgs1 and Dna1 as well as other partners...

Referee #2:

In this manuscript, Kasaciunaite and colleagues developed a detailed mechanistic description explaining coordination between the components of the yeast long-range DSB processing machinery.

Single-molecule analysis of the DNA substrates stretched by the magnetic tweezers allowed the authors to characterize the duplex unwinding behavior of Sgs1 helicase. Like other previously studied RecQ family helicases, Sgs1 unwinds duplex DNA containing a 5' flap structure moving in a 3'-5' direction. The same Sgs1 molecule undergoes several unwinding bursts per binding event. The forward unwinding is followed by a rapid duplex rezipping or strand switching and slow translocation. RPA, another component of end resection reduces the rates of Sgs1-unwinding and rezipping. This likely takes place through an allosteric interaction between the two proteins - the experiment at different forces is quite convincing.

Similar analysis was previously used by the authors to visualize duplex unwinding by a nuclease deficient Dna2 helicase/nuclease in the presence of RPA. Here, the authors proceeded to combine the three components of the DSB long resection and analyzed the DNA unwinding/degradation by Sgs1/Dna2 and RPA together. The signature of the unwinding events allowed the authors to propose a very interesting and quite plausible model whereby Sgs1 unwinds the duplex by moving on the 3'-terminated strand, while Dna2 is engaging the other strand either mobbing in 5'-3' direction (nuclease mutant) or digesting the 5' end (helicase mutant or w.t. protein). The latter provides shorter and shorter distance for the Sgs1 backtracking. This is a nice and timely addition to our knowledge of the DSB processing in eukaryotes, which is so critical to accurate DNA repair. The cooperation between the two helicases and their physical assembly into the complex (the salt experiment was a really good control) resembles that of the bacterial end resection machine, which suggest a universal mechanism of HR initiation.

Overall, the experiments presented here are cleverly designed and expertly performed. The results support the authors' conclusions. The model the authors developed is sound and will be of a significant interest to readers. There are a few points, however, that authors need to address to strengthen their interpretation and model:

1. The authors state that based on their previous work Sgs1 unwinds DNA 2x faster than Dna2. While this is true on average, the distribution in ref 13 looks like a sum of two pools of unwinding rates, very slow and a broadly distributed fast molecules. Moreover, this distribution of the rates of unwinding by a nuclease deficient mutant. In this current work the authors use the w.t. Dna2 along with its helicase and nuclease mutants. It would be proper if the distribution rates for these enzymes are analyzed and presented in the manuscript. Currently, one can see only one trace (inset in Fig. 3A), and this one trace is of a quite fast molecule. The authors also state that their observed similar unwinding behaviors by the complexes containing Dna2 and its nuclease and helicase mutants. These data need to be prominently displayed.
2. The authors suggest an allosteric regulation of Sgs1 by RPA. This is quite likely, but for an unambiguous determination it would be great to see an experiment with an Sgs1 mutant deficient in RPA binding, such as described in ref 25.
3. It is not very clear from the model if the authors envision that a single RPA molecule (or several molecules) affects the helicase activity and is/are incorporated into the complex with Sgs1 and Dna2, or whether Sgs1 transiently interacts with and releases multiple ssDNA bound RPAs. Please discuss.

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In their manuscript, Kasaciunaite and coauthors present an exciting study of the 'cooperative' action of the different player in the 5' end-resection phase of HR-mediated DNA double-strand repair in yeast. They employ magnetic tweezers to show that RecQ helicase Sgs1 and helicase-nuclease Dna2 activity are substantially affected by single-stranded binding proteins RPA and Top3-RMI1. Overall, the study addresses (and answers) an important question and is performed in a sound way, with the right amount of control experiments. I think this manuscript should be published in EMBO Journal. Below, I mention a couple of points that the authors could use the further improve the manuscript.

Contents wise I had one point and that concerns the stoichiometry of the different proteins. The authors can not (do not) check this, other than changing the concentration of added proteins. Fair enough. It might, however, be useful to discuss what is expected here, how intermittent interactions are (due to individual proteins binding, falling off).

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p. 8 halfway "...indicates the progressive...". I could not follow the logic of reasoning & sentence here.

Point-by-point response to referee remarks

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We note that the assay which monitors DNA unwinding resulting from its extension associated with dsDNA to ssDNA conversion is well established and has been applied to other enzyme systems before by multiple groups (Wuite et al, 2000, Maier et al, 2000, Kemmerich et al, 2016, Dessinges et al, 2004, Levikova et al, 2013). That said, we agree that the description may have been insufficient for non-experts.

We modified the text and added a new Fig S1 to the Appendix with additional explanations in the figure caption, which explain that DNA lengthening we observe is due to ssDNA production, i.e. dsDNA unwinding. Effectively, dsDNA (the original form of the molecule before unwinding by a helicase occurs) has a characteristic extension behavior when increasing forces are applied. When the double-stranded DNA gets unwound such that the force-bearing part of the molecule becomes ssDNA, then the characteristic force-extension behavior changes. Above forces of 5 pN, the extension of ssDNA is (for the same number of nucleotides per strand) larger than that of dsDNA. During DNA unwinding at a fixed force >5 pN we therefore see a DNA length increase (Fig S1B). We also test single-strand DNA formation regularly by fitting the force-extension data after unwinding with well-established polymer models (Fig S1B). Since we start our measurements with a practically intact dsDNA molecule (containing only a short 38 nt ssDNA gap), the large length increases we observe during unwinding cannot be due to fold-back structures. Foldbacks would be anyway already resolved before adding the helicase at applied forces of > 15 pN. Nicking would rather lower the DNA extension by decreasing its bending rigidity. We also know from gel-based experiments that our protein preparations are free from contaminants and do not nick DNA.

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We made attempts to improve the English. Responses to specific comments are appended below.

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- P5, Consistently, no DNA length.....when omitting ATP or protein in the reaction (data not shown).

The requested control experiments were added as a new Fig S2 to the Appendix. No helicase activity (DNA lengthening nor shortening) was seen when omitting either ATP, protein or both components from the reaction.

- P5, Since the rate of these sections was approximately constant and was on the similar? order of magnitude of the unwinding rate, we attribute..... (not shown)

The corresponding data was actually already shown in the histograms of Fig 1C (compare lower and upper panel). We now additionally refer to the lower panel of Fig 1C on page 5 to clarify this point.

- *P7, A similar skew was also observed at elevated RPA concentration (50 nM)for the observed rate distribution (data not shown).*

We added the histograms of the rate distributions as well as the skewness at 50 nM RPA to Fig EV2.

- *P8, Interestingly, upon removing the excess.....(i.e. no reziping occurred) (data not shown)*

We added the corresponding time trajectory as a new Fig S5 to the Appendix. It shows that after DNA unwinding by Dna2, the amount of unwound DNA does not change when flushing the fluidic cell with 3M NaCl. We refer to this trajectory on page 8 of the main text.

- *P10, Independent of the presence of Top3-Rmi1,.....in the absence of RPA (data not shown)*

The requested control experiments were added as a new Fig S8 to the Appendix. In the absence of RPA no DNA unwinding by Sgs1 was detected at high salt conditions. In the presence of Top3-Rmi1 occasionally a sudden step-wise DNA length increase was seen, which can be attributed to the ssDNA cleavage activity by Top3-Rmi1 (see Fig. EV3). This suggests that the Top3-Rmi1 activity was not impaired by the higher salt concentrations. However, it appears that the protein fails to stimulate the recruitment of Sgs1.

- *At high salt conditions RPA was essential for activity....(data not shown)*

As demonstrated by the controls added to Fig S8, no unwinding activity was observed in the absence of RPA at high salt conditions. In the presence of RPA, unwinding was, however, observed (Fig EV4). Thus, RPA was essential for DNA unwinding by Sgs1 at high salt. To additionally illustrate this, we added a trajectory as a new Fig S9. Here, we show that initially ATP and Sgs1 did not provide DNA unwinding, while a subsequent addition RPA (on top of Sgs1 and ATP) to the same DNA molecule resulted in unwinding.

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We carried out the requested control experiments in the absence of RPA and added them as a new Fig S7 to the Appendix. Dna2 wt alone does not show any unwinding activity in the absence of RPA. For Sgs1 and Dna2 wt in the absence of RPA, we observed only the typical Sgs1 unwinding activity with fast reziping of the unwound DNA and return to the original baseline level of full-length dsDNA, i.e. no persistent unidirectional DNA processing. We added an additional sentence on page 9 to refer to this figure.

2. *Strand switching by Sgs1 could also be a way to limit excessive resection by Dna2 by inducing a local block. This should be tested by checking whether helicase dead Dna2 resection processivity is altered by Sgs1*

We analyzed total DNA unwinding by Dna2 alone as well as in complex with Sgs1 that was achieved during the observation period of 30 min (all reactions in the presence of RPA). This result was added as a new Fig S6. No significant difference was observed between nuclease dead Dna2 (Dna2 675) alone and the reactions containing Sgs1 as well as wt, nuclease-dead, helicase-dead Dna2 or nuclease-helicase-dead Dna2. Thus, the reziping activity of Sgs1 does not provide a roadblock for Dna2. We added a sentence on page 9 to point out the similar processivities of the progressive DNA unwinding.

3. *They need to test the DNA duplex processing with Sgs1-r1 mutant protein (ref 25, Hegnauer et al., EMBO J, 2012), which lacks a major RPA binding domain. This will confirm the statement that RPA modulates SGS1 activity through protein interaction, not simply as roadblock. Testing this mutant would also allow one to evaluate the in vivo impact of their in vitro studies, because this mutant was extensively studied in vivo.*

The mentioned reference identified a major interaction site of RPA within Sgs1, which lies between amino acids 404 and 604. Its deletion is likely to affect also other properties of the enzyme, such that obtained data may be less conclusive than one hopes for. For time constraints in preparing the revision (mutant cloning + purification > 2 months) and since this mutant has so far not been purified *in vitro*, we rather used the Sgs1 641-1215 fragment that was already available in our laboratory. It contains the helicase core of Sgs1, which is known to be capable of DNA unwinding. A similar fragment of BLM is also frequently used in biochemical analyses (Janscak et al, 2003). This helicase core also lacks the major RPA interaction site. Our single-molecule measurements reveal a similar pattern of DNA unwinding as observed with wt Sgs1 with dominant fast re-zipping in the absence and slow rewinding in the presence of RPA (see new Fig S3 in the Appendix). Importantly, in the presence of RPA no skew of the distribution towards lower unwinding velocities was observed. This supports that specific interactions with RPA cause the skew of the velocity distribution towards lower values for wt Sgs1. The distributions of the rewinding velocities are similar in presence and absence of RPA though being broader as also observed for wt Sgs1.

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We tested the suggested nuclease- and helicase-dead double mutant, which we just had purified. Interestingly, the double mutant did also promote a progressive unwinding in the presence of RPA and Sgs1 (new Fig EV1D). One possibility to explain this behavior is an external binding (without threading onto the DNA molecule as identified by (Zhou et al, 2015) of the Dna2 mutant to the DNA unwound by Sgs1 at random positions. These randomly bound Dna2 molecules could act as road blocks and prevent DNA rewinding by Sgs1. To test this possibility, we mechanically induced DNA unwinding in the presence of the double mutant (new Fig EV1E, EV1F). Subsequent lowering of the force allowed DNA rewinding. The observed rewinding always fully converted all unwound DNA to dsDNA, i.e. Dna2 did not function as a ratchet under these conditions, excluding the possibility of external binding. Therefore, Sgs1 is required to induce progressive DNA unwinding in the presence of the double mutant. We added this rather surprising observation to the text on pages 8-9. Furthermore, we suggest a ratchet mechanism to explain how the mutant can contribute to progressive DNA unwinding, which is included in Discussion on page 15. We are not aware that such a ratchet mechanism has been described for another protein to date. To fully elucidate this in our opinion very interesting mechanism is however beyond the scope of this study. Due to the stringent requirement for DNA unwinding by Sgs1, we can exclude that we observe an unspecific effect (e.g. random protein binding), and our general conclusions thus remain valid.

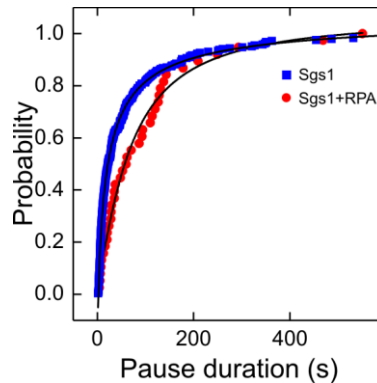
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The reviewer is correct that the different possibilities have been discussed in depth in previous research. Particularly in the work on AtRecQ2 of our group we provide evidence for a strand-switching mechanism rather than a back-sliding. In the current manuscript we are quite careful to not overstate the strand switching and refer to the previous works: "Due to the functional similarities between Sgs1, BLM and AtRecQ2 (Oh et al, 2007, De Muyt et al, 2012, Klaue et al, 2013), we suggest that Sgs1 also undergoes cycles of strand switches during repetitive DNA unwinding-re-zipping events".

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probably reflected in different probability distribution. Some probability analysis is needed to support the claim.

Generally the cumulative pause times are exponentially distributed in line with single-complex binding (see attached plot). However, due to limitations of the single-molecule statistics (fewer bursts compared to individual events), a sigmoidal behavior indicating cooperativity cannot be fully excluded. Even for a pure exponential distribution of cumulative pause times multi-protein binding cannot be excluded if the rates differ. We therefore did not include this data in the manuscript and rather toned down our initial statement on page 5.



Minor points:

- *Why is it stated that the histograms are presented {plus minus} SEM ? (Except Fig 5)*

We removed these statements that referred to previous figure versions where the mean and the S.E.M. was given in the figure.

- *English needs help here: "however the underlying interplay of this interaction remains undefined." In introduction "mandate cognate RPA" (introduction page 4) Please improve English. Intro last para: (A)lthough functional synergies...., however, how Sgs1 and Dna1 as well as other partners...*

We improved the wording in the mentioned sentences. Also we went carefully through the manuscript and improved the English at several additional spots.

Referee #2:

In this manuscript, Kasaciunaite and colleagues developed a detailed mechanistic description explaining coordination between the components of the yeast long-range DSB processing machinery. Single-molecule analysis of the DNA substrates stretched by the magnetic tweezers allowed the authors to characterize the duplex unwinding behavior of Sgs1 helicase. Like other previously studied RecQ family helicases, Sgs1 unwinds duplex DNA containing a 5' flap structure moving in a 3'-5' direction. The same Sgs1 molecule undergoes several unwinding bursts per binding event. The forward unwinding is followed by a rapid duplex rezipping or strand switching and slow translocation. RPA, another component of end resection reduces the rates of Sgs1-unwinding and rezipping. This likely takes place through an allosteric interaction between the two proteins - the experiment at different forces is quite convincing.

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Processive DNA unwinding by Dna2+RPA (in the absence of Sgs1) was only seen for the nuclease-dead mutant. Both for wt Dna2 and for helicase-dead Dna2 no activity was seen in a helicase assay. This was already reported by Levikova et al, 2013 and could be attributed to the nuclease activity of these enzymes, which would degrade the 5'-flap of the DNA that is required for the helicase loading. To make this point clearer, we added a new Fig S4 to the Appendix, in which we show trajectories of the DNA length recorded for wt, helicase-dead and nuclease-dead Dna2. The former two variants do not exhibit any DNA unwinding, while for the latter the typical Dna2 unwinding was seen. We therefore cannot measure the unwinding rate distributions for wt and helicase-dead Dna2 in the absence of Sgs1. We provide however a rate distribution for the nuclease-dead variant in Fig S4D. Only in combination with Sgs1 (as displayed in Fig EV1) the Dna2 mutants (including new data for the double mutant of Dna2) produce similar progressive DNA unwinding as wt Dna2.

- 2. The authors suggest an allosteric regulation of Sgs1 by RPA. This is quite likely, but for an unambiguous determination it would be great to see an experiment with an Sgs1 mutant deficient in RPA binding, such as described in ref 25.*

A similar question was raised by referee 1 (see point 3), such that we use the same response: The mentioned reference identified a major interaction site of RPA within Sgs1, which lies between amino acids 404 and 604. Its deletion is likely to affect also other properties of the enzyme, such that obtained data may be less conclusive than one hopes for. For time constraints in preparing the revision (mutant cloning + purification > 2 month) and since this mutant has so far not been purified *in vitro* we rather used the Sgs1 641-1215 fragment that

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As suggested, we added a brief section to the discussion on page 14: “The stoichiometry of this complex remains to be determined. We hypothesize that it contains a single Dna2 (Zhou et al, 2015), a monomer or a dimer of Sgs1 (Cejka et al, 2010) and a low number of RPA molecules. The latter may also make contacts to the unwound ssDNA and may be dynamically exchanged, e.g. be loaded or removed to or from ssDNA.”

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We added additional cartoons as suggested by the referee to portray a clearer view on the activity of the different proteins.

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This has been corrected as suggested.

- p. 7 halfway. I do not completely understand what the barrel-shape of Dna2 has to do with it unidirectionally unwinding DNA. Maybe more info could be added to make it clearer or this part could be left out, since it does not seem to be so important (here).*

Previous structural studies showed that the protein has a cylindrical shape with a central tunnel through which the ssDNA threads (Zhou et al, 2015). Most of the DNA-binding sites are inside the tunnel, creating multiple bonds with DNA phosphodiester groups as well as the main chain. Since the nuclease domain is practically encircling the DNA, it should only proceed forward or potentially slip back but not dissociate, explaining the high processivity of DNA unwinding by Dna2. In our previous (Levikova et al, 2013) and also this study we did not observe Dna2 moving backwards. We added a better explanation of the tunnel-forming structure of Dna2 on page 3 and better explain on page 7, why this structure should prevent rewinding.

- p. 8 halfway "...indicates the progressive...". I could not follow the logic of reasoning & sentence here.*

When preparing the revised manuscript we found that the double-mutant of Dna2 (helicase-dead and nuclease-dead) can also induce a progressive unwinding as seen for the other Dna2 variants. We therefore made larger changes on page 8 and also modified the sentence that was unclear to the reviewer (see also our reply to point 4 of referee 1).

- Cejka P, Cannavo E, Polaczek P, Masuda-Sasa T, Pokharel S, Campbell JL, Kowalczykowski SC (2010) DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2. *Nature* **467**: 112–116
- De Muyt A, Jessop L, Kolar E, Sourirajan A, Chen J, Dayani Y, Lichten M (2012) BLM Helicase Ortholog Sgs1 Is a Central Regulator of Meiotic Recombination Intermediate Metabolism. *Mol Cell* **46**: 43 – 53
- Dessinges MN, Lionnet T, Xi XG, Bensimon D, Croquette V (2004) Single-molecule assay reveals strand switching and enhanced processivity of UvrD. *Proceedings of the National Academy of Sciences* **101**: 6439–6444
- Janscak P, Garcia PL, Hamburger F, Makuta Y, Shiraishi K, Imai Y, Ikeda H, Bickle TA (2003) Characterization and Mutational Analysis of the RecQ Core of the Bloom Syndrome Protein. *Journal of Molecular Biology* **330**: 29 – 42
- Kemmerich FE, Daldrop P, Pinto C, Levikova M, Cejka P, Seidel R (2016) Force regulated dynamics of RPA on a DNA fork. *Nucleic Acids Res* **44**: 5837–5848
- Klaue D, Kobbe D, Kemmerich F, Kozikowska A, Puchta H, Seidel R (2013) Fork sensing and strand switching control antagonistic activities of RecQ helicases. *Nat Commun* **4**: 2024
- Levikova M, Klaue D, Seidel R, Cejka P (2013) Nuclease activity of *Saccharomyces cerevisiae* Dna2 inhibits its potent DNA helicase activity. *Proc Natl Acad Sci* **110**: E1992–E2001
- Maier B, Bensimon D, Croquette V (2000) Replication by a single DNA polymerase of a stretched single-stranded DNA. *Proceedings of the National Academy of Sciences* **97**: 12002–12007
- Oh SD, Lao JP, Hwang PYH, Taylor AF, Smith GR, Hunter N (2007) BLM Ortholog, Sgs1, Prevents Aberrant Crossing-over by Suppressing Formation of Multichromatid Joint Molecules. *Cell* **130**: 259 – 272
- Wuite GJL, Smith SB, Young M, Keller D, Bustamante C (2000) Single-molecule studies of the effect of template tension on T7 DNA polymerase activity. *Nature* **404**: 103
- Zhou C, Pourmal S, Pavletich NP (2015) Dna2 nuclease-helicase structure, mechanism and regulation by Rpa. *eLife* **4**: e09832

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below). I am pleased to say that the referees find that their comments have been sufficiently addressed and now support publication. Nonetheless referee #1 still raises two points, the first of which will require textual changes only, and should be addressed in the revised version. As for the referee's second comment on the incorporation of the EV figures, given that these are also displayed in line in the online version, we will not require you to move everything into the main figures. However, we do encourage you to add some crucial controls and experiments important for understanding to the main figures.

REFEREE REPORTS

Referee #1:

The paper is improved. I have 2 serious criticisms:

- 1) The authors write that Dna2 and Sgs1 and cofactors are forming a complex but based on the data shown, they only influence each others activity at the same lesion. That doesn't mean that they are a complex. This speculation should be removed or be declared a speculation.
2. All the Extended data figures except the last one, **MUST** be in the paper main figures. They are essential to make to paper convincing. The last can be supplemental.

This second recommendation is crucial for making the paper competitive.

Referee #2:

The authors fully addressed this reviewer's previous concerns. The inclusion of the Sgs1 core, which lacks RPA interaction is an important control and further strengthens the conclusions. I believe this study along with the model proposed by the authors will be of an interest to the EMBO J readership.

Regarding comments from Referee #1:

1) The authors write that Dna2 and Sgs1 and cofactors are forming a complex but based on the data shown, they only influence each others activity at the same lesion. That doesn't mean that they are a complex. This speculation should be removed or be declared a speculation.

REPLY: We modified the text as suggested. Specifically, we use "hypothesize" or similar when we mention complex formation. However, physical interactions between the respective factors (Dna2 with Sgs1, Sgs1 with RPA, Sgs1 with Top3-Rmi1) were demonstrated in previous studies, and we include the references in support. That said, the reviewer is correct that we do not know how much "complex" was formed under our conditions, and we thus agree with these modifications.

*2. All the Extended data figures except the last one, **MUST** be in the paper main figures. They are essential to make to paper convincing. The last can be supplemental.*

REPLY: We think that our main figures cut to the chase and help the reader focus on the main points. The EV figures are mostly controls or repetitions carried under different conditions, which conceptually do not advance the main message. Therefore, for sake of clarity, we would prefer to leave the main vs. EV figures divided as they were.

Accepted

8th May 2019

Thank you for submitting you for submitting the final revised version of your manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in *The EMBO Journal*.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ralf Seidel
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-101516R

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 χ^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?	No statistical method was used to determine sample size. However, sample sizes were chosen to ensure reproducibility of experiments while ensuring the validity of effects described in the paper.
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?	In general no data have been excluded. However, in case of obvious technical issues the data were not considered for analysis.
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.	NA
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.	NA
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5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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<http://www.antibodypedia.com>
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<http://www.consort-statement.org/checklists/view/32-consort/66-title>

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<http://figshare.com>

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

<http://biomodels.net/>

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<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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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).	NA
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

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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
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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
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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'. 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	Data is available from the corresponding authors upon reasonable request.
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
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