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An N-terminal acidic region of Sgs1 interacts with Rpa70 and recruits Rad53 kinase to stalled forks

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1st Editorial Decision 07 February 2012

Thank you for submitting your manuscript on the Sgs1-Rpa70 interaction for consideration by The EMBO Journal. We have now received the reports from three expert reviewers, which are copied below. These referees all acknowledge the importance of the topic and also the potential interest of your new findings, but nevertheless raise a number of issues that would need to be satisfactorily addressed before publication may be warranted. These issues include points in need of clarification and aspects of presentation/interpretation, however there are also more substantive concerns regarding the depth of insight and overall advance conveyed by the results at the current stage. In this respect, especially referee 3 offers several constructive and well taken experimental suggestions to deepen the insights that can be gained from the study, and which appear to be well within the scope of the current analysis. We therefore feel that the manuscript, despite the presently somewhat cautious referee enthusiasm, may become a stronger candidate for EMBOJ publication if extended along the lines suggested by the reviewers, in particular referee 3. Thus, should you be able add these requested experiments as well as to address the various more specific issues raised by all reviewers, than we should be able to consider a revised manuscript further for publication.

Please be reminded that it is our policy to allow a single round of major revision only, and that it will therefore be important to diligently and comprehensively answer to all the points raised at this stage in the process. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this work, Hegnauer and colleagues investigated to what extent the reported association of Sgs1 with RPA would mechanistically correlate to the observed phenotype of sgs1-delta strains after replication fork stalling. To this end, the authors undertook biochemical and genetic analyses to map the interaction site of Sgs1 with RPA70 and then generated interaction mutants to investigate the phenotype after fork stalling. Moreover, they performed functional analysis of the cross-talk between Sgs1-RPA and Rad53 during the activation of the S-phase checkpoint.

The mechanisms controlling that stalled replication forks are properly stabilized and recovered are of paramount importance for the maintenance of genome integrity. Moreover, they are thought to represent powerful barriers against cancer progression in humans. RecQ helicases, WRN and BLM in particular, are central to the stability of genomes during DNA replication and studies aiming to unveil how they perform this function are very important in the field.

From this point of view, the work of Hegnauer and colleagues is clearly a significant improvement over what we know about the function of Sgs1 (and by extension that of human orthologues) and is also a step forward our understanding of how stalled works are stabilized when perturbed or stalled. Experiments are well described and performed and conclusions are generally well supported by the results. The ms is well written, even though I suggest authors to try reducing the length of the discussion, that is a little bit wordy in some sections.

I have just three minor comments on the manuscript and one suggestion:

1. Figure 6A. The WB included in the ms should be improved, though the results can be already appreciated, a clearer blot could help in making easier the assessment of the behavior of the mutants.

2. The reported function of the Mec1 phosphorylation site in regulating the interaction of Sgs1 with Rad53 is very interesting. However, earlier works showed that deletion of Rad53 leads to enhanced Rad51-dependent recombination. The authors have any data on Rad51 chromatin recruitment in their mutants where Rad53 gets poorly activated? At least, the authors should try to discuss this possibility.

3. A recent paper demonstrated ATR-dependent phosphorylation of the human RecQ helicase WRN. In that context, ATR-dependent phosphorylation would prevent fork inactivation. It seems that association with RPA of Sgs1 could help doing the same job. The authors should evaluate to briefly discuss about similarities and differences between yeast and human RecQ helicases as for what concerns maintenance of stalled fork stability. Indeed, also WRN has been showed to associate with RPA but the functional significance of this interaction, in vivo, remains elusive also because interaction has been mapped both at the N and the C-terminal portions of the protein.

Last, my suggestion is to evaluate whether the sgs1 mutant that cannot associate with Rad53 shows any defect in downstream checkpoint signaling or, at least, perform cell cycle analyses. The mutants generated by the authors are very intriguing and could help in clearly uncover distinct Rad53-regulated events, so a little bit of more insights would make the work even more interesting.

Referee #2 (Remarks to the Author):

The manuscript "Sgs1-Rpa70 interaction domain stabilizes DNA polymerase and recruits Rad53 kinase to stalled forks" by Hegnauer et al describes a separation-of-function allele of Sgs1. The interaction domain between Sgs1 and RPA-70 was mapped where deletion of Sgs1-404-604 reduced RPA association two-fold. The authors suggest that there may be additional RPA binding sites as well since this reduction was partial and rather weak (especially in the supplemental Figure 2). This region also binds to the FHA domain of Rad53 by yeast-2-hybrid. In this sgs1-r1 mutant, polymerase association to an early origin was epistatic to mec1-100. The authors conclude that RPA-interacting domain is responsible for Sgs1 interaction of Mec1, which would phosphorylate Sgs1 enabling Rad53 recruitment and activation of the DNA damage checkpoint during replication fork arrest caused by HU. The link between Rpa70 interaction with Sgs1 in the same region as DNA polymerase and the Rad53 kinase is not connected well in the manuscript. It seems like they are distinct observations. The authors suggest a model in Figure 7B that show a competition between RPA and Rad53 for Sgs1 binding but no data is shown to support this model. It would be essential to show data to support this model if it is to be highlighted so prominently in the manuscript. Overall the manuscript adds to our understanding of Sgs1 during DNA replication by pinpointing different regions of Sgs1 that are separate from its helicase and other known domains.

Major comments:

1. In Figure 2A, the authors describe testing the Sgs1-B42-AD deletion constructs lacking different sequences (either 404-485, 496-536, or 565-604) based on sequence conservation and conclude that the first two sites are needed for RPA70 binding. It is not clear how these sites are defined and since they very closely overlap with some previously defined acidic regions, it would be helpful to have the conserved sequence alignments shown. It's not obvious why 404-485 and 496-536 are really two distinct sites and not one.

2. In Figure 3A, the Sgs1-r1-13MYC appears to be expressed better than the endogenous Sgs1-13MYC and not at comparable WT levels.

3. In Figure 4, it is a weak argument that Sgs1-RPA interaction contributes to DNA pol binding at stalled forks when it was observed for one early origin but not a later firing origin. Additional origins of replication should be analyzed (such as the early origin ARS305 and a corresponding late origin).

4. It is unclear how ITC experiments involving RPA suggest a phosphorylation-independent pathway for interaction between RPA and Sgs1, which is asserted in the discussion in support of Figure 7B. The rationale for these conclusions should be expanded if Figure 7B is to remain part of the manuscript.

5. On p. 13 the authors state "We found that Sgs1(404-604) is a very robust substrate for Mec1-Myc phosphorylation, being phosphorylated better than target peptides from Rad53 (Fig. 5C)." It is not clear what target Rad53 peptides are shown in this figure. Also in Figure 5C, why is the untagged Mec1 not phosphorylating the WT Sgs1-404-604 since this band is not sensitive to caffeine like the tagged version.

6. The competition model shown in Figure 7B between RPA and Rad53 binding is speculative and should not be part of the model shown. It could be discussed as a possibility but there is no evidence for that model in the data shown. Experiments such as an RPA yeast-2-hybrid assay with mutated phosphoacceptor sites on Sgs1 (similar to figure 5C) could be performed, or competition assays with RPA and Rad53 for Sgs1 binding if the authors want to test this hypothesis.

Minor comments:

1. Figure 7 is overly complicated, and could be simplified to highlight the more important parts of the proposed model for the role Sgs1 interactions in replisome stability.

2. Figure 4 is a difficult figure for the reader, as the figure legend is presented only once and is disjointed.

Referee #3 (Remarks to the Author):

In this manuscript, Hegnauer et al. explore the physical interactions of the RecQ helicase Sgs1 with RPA and Rad53, and attempt to determine the significance of these interactions for fork stabilization and checkpoint activation upon replication stress. The authors, and others, have previously shown that:

a. Sgs1 and RPA interact (Cobb Embo J '03)

b. That Sgs1 and Rad53 interact, and have mapped this interaction to the central region of Sgs1, and Rad53's FHA phospho-epitope binding domain. (Bjergbaek Embo J. '05)

c. Sgs1 is important for Rad53 phosphorylation in cells lacking RAD24 (Frei G&D '00)

d. The human homologues of Sgs1 are ATR/ATM targets (Davies, Pichierri, Rao)

In this manuscript, the authors do two things. First (figures 1 & 2 & 5D) they define the interaction domains more precisely, and determine the Kd and stoichiometry of each interaction. This work is well-done and valuable, but doesn't provide much biological insight. Next, the authors show that Sgs1 is a target of Mec1 in vitro, and suggest that Mec1 phosphorylation of Sgs1 promotes the Sgs1-Rad53 interaction, which results in Rad53 recruitment to stalled replication forks. While the previously published data had already led the authors to suggest that Sgs1 recruits Rad53, and thus function in checkpoint activation, this manuscript goes further by suggesting that the recruitment is mediated directly by Mec1. The problem here is that the authors have not shown that this phosphorylation occurs in vivo, or that it promotes Rad53 binding and recruitment to forks in vivo. Therefore, this paper is not sufficiently novel for publication in EMBO without significant additional experiments to validate the claims made in the last half of the paper.

Suggested experiments:

1. Try to show a phenotype for the sgs1-4A mutant. In Figure 6A, the sgs1-4A rad24 double mutant does not seem to have a significantly smaller Rad53 shift than the rad24 . This result could be made clear using a more-quantitative assay such as ISA. Alternatively, if tel1 were introduced into all of the strains, the sgs1-4A rad24 tel1 might show no Rad53 shift at all. If this strain still shows Rad53 phosphorylation, introduction of mrc1-aq might eliminate it (if Sgs1 and Mrc1 play redundant roles in Rad53 recruitment).

2. Similarly, in Figure 6B the sgs1-4A mutant has no survival defect in long-term growth on HU (not surprising given the result for sgs1-r1 in Figure 3E). The sgs1-4A mutant should be tested for recovery from acute HU stress as in Figure 4A. This should be done in combination with both rad24 and rad53 . Their data suggests that rad24 will help uncover the redundant Rad53 activation phenotype. Importantly, if the sgs1-4A allele is specifically defective for Rad53 recruitment, as opposed to generally hypomorphic, it should be completely epistatic to rad53 (perhaps in rad24).

3. Show that Sgs1 is phosphorylated by Mec1 in vivo. There are several potential ways to do this:
A. Mass spec on Sgs1 digesting with an enzyme other than trypsin (the authors note that the tryptic fragment is too large).
B. Showing a Mec1- and damage-dependent gel shift for Sgs1, possibly using Phostag to exaggerate a small shift.
C. IP-ing Sgs1 and then western blotting it with the Cell Signaling anti-S/TQ antibody.
D. Making a phospho-specific antibody to one of the 4 sites mapped on Sgs1.

4. Show that Mec1 phosphorylation of Sgs1 promotes the Sgs1-Rad53 interaction in vivo. The most obvious way to do this is to show that Sgs1-wt and Rad53 co-IP, but sgs1-4A and Rad53 do not. If this is not possible, the relatively modest difference between Sgs1-wt and sgs1-4A interaction with Rad53 by yeast two-hybrid (Figure 5E) might be made more convincing by showing that it is dependent on Mec1/Tel1. That is, the authors should repeat both the wt and the 4A in both MEC1 and mec1 strains, and show that mec1 mutants have a lower 2 hybrid, and that this is epistatic to 4A. It should be noted that the model that this association is damage driven appears at odds with Fig 2A of the authors 2005 Embo paper, which shows that the Sgs1 Rad53 co-ip is not affected by DNA damage. This needs to be addressed.
Major concerns:
5. In Figure 3C, there is significant binding of RPA to beads alone, calling into question the result. Also, the proper negative control is a strain in which Sgs1 is not tagged, not no antibody.

6. Figure 4C and D are used to argue that mec1-100 is epistatic to sgs1-r1. However, as in Figure 3E (for which the authors properly note that epistasis cannot be established), there is no convincing defect in the sgs1-r1 single mutant. Moreover, the conclusion of the epistasis is not spelled out. If mec1-100 is epistatic, does that mean that it is required for the Sgs1-RPA interaction?

Minor concerns:
7. In Figure 3E, the epistasis results might be more clear if mec1 were used in addition to mec1-100.

8. In the references in the text to Figure 4, it is suggested that the observed results reflect the disrupted interaction with RPA in the sgs1-r1, but they could as easily reflect a disruption of the Sgs1-Rad53 interaction.

9. In the paragraph that begins, "Because HU-treatment...," it should be stated that mec1-100 is epistatic to sgs1-r1, not the other way around.

10. Since all tested phenotypes of sgs1-r1-hd are similar to sgs1-hd, it is unclear what conclusions can be drawn.

1st Revision - authors' response 29 May 2012

Major additions and changes to Hegnauer et al:

1. We have shown that Sgs1 is phophorylated in vivo and have mutated the acceptor sites. We see a Sgs1 phosphoshift after "standard" HU treatment (0.2 M, for 60 min), from which we have phosphosite analysis by MS. Unfortunately, the crucial Mec1 target (T451) is in a fragment we cannot analyse by MS. Nonetheless, we lose the upshift with a point-mutated form of Sgs1 that eliminates the TQ acceptor sites. Moreover, we show that the modification is Mec1 dependent in vivo (and not just in vitro).

2. We improved the Rad53 phosphorylation assay, and show that both the *sgs1*-r1 and *sgs1*-4A mutants have equally strong effects on Rad53 upshift.

3. Recovery from HU-arrest assays were repeated for all strains and double mutants. We found that one of our *sgs1*-r1 strains had a secondary mutation, which has now been remedied. The *sgs1*-4A mutant is in a supplemental figure – neither has a fork recovery defect on HU.

4. We included results from a synthetic lethal screen for synthetic effects with sgs1-r1, and we confirmed the effects with double mutants on plates containing DNA damaging agents. We tested *sgs1*-r1 double mutations with various recombination mutants in the E-MAP analysis, but there was no strong synergy with *sgs1*-r1.

5. We used CHIP to analyse three more origins for polymerase presence on HU, in appropriate mutants.

6. We confirmed and improved controls for the pull-down experiments with Sgs1 and RPA and with Rad53.

7. We identified and confirmed phenotypes for these mutants on other DNA damaging agents.

Detailed replies to reviewers:
Referee #1 (Remarks to the Author):

In this work, Hegnauer and colleagues investigated to what extent the reported association of Sgs1 with RPA would mechanistically correlate to the observed phenotype of *sgs1*-delta strains after replication fork stalling. To this end, the authors undertook biochemical and genetic analyses to map the interaction site of Sgs1 with RPA70 and then generated interaction mutants to investigate the phenotype after fork stalling. Moreover, they performed functional analysis of the cross-talk between Sgs1-RPA and Rad53 during the activation of the S-phase checkpoint.

The mechanisms controlling that stalled replication forks are properly stabilized and recovered are of paramount importance for the maintenance of genome integrity. Moreover, they are thought to represent powerful barriers against cancer progression in humans. RecQ helicases, WRN and BLM in particular, are central to the stability of genomes during DNA replication and studies aiming to unveil how they perform this function are very important in the field.

From this point of view, the work of Hegnauer and colleagues is clearly a significant improvement over what we know about the function of Sgs1 (and by extension that of human orthologues) and is also a step forward our understanding of how stalled works are stabilized when perturbed or stalled.

Experiments are well described and performed and conclusions are generally well supported by the results. The ms is well written, even though I suggest authors to try reducing the length of the discussion, that is a little bit wordy in some sections.

Discussion was shortened

I have just three minor comments on the manuscript and one suggestion:

1. Figure 6A. The WB included in the ms should be improved, though the results can be already appreciated, a clearer blot could help in making easier the assessment of the behavior of the mutants.

We have repeated the Rad53 phosphorylation experiment including one more time point (0, 30, 60 min) and we consistently observe reduced Rad53 phosphorylation in *sgs1*-r1 rad24, *sgs1*-4A *rad24* mutants at 60 min. This is now shown in Figure 6E.

2. The reported function of the Mec1 phosphorylation site in regulating the interaction of Sgs1 with Rad53 is very interesting. However, earlier works showed that deletion of Rad53 leads to enhanced Rad51-dependent recombination. The authors have any data on Rad51 chromatin recruitment in their mutants where Rad53 gets poorly activated? At least, the authors should try to discuss this possibility.

Unfortunately, tagging Rad51 on either N- or C-terminus renders it non-functional, and despite many attempts, we were unable to locate an anti-Rad51 Ab that works for CHIP. Instead, we took a genetic approach and performed *sgs1* and *sgs1*-r1 EMAP analysis with ~1600 nuclear and chromatin associated mutants (including recombination mutants). We checked for genetic interaction with Rad51 (Fig S6) and both replication and recombination mutants (Figure 4), but found no strong genetic interactions with *sgs1*-r1. Rather, we found and confirmed strong negative genetic interaction with *mus81*Δ (a structure specific endonuclease). This argues that *sgs1*-r1 may be defective in dissolution of recombination intermediates. Consistently we found that *sgs1*-r1 is sensitive to Zeocin (Figure 6F). Since the *sgs1*-4A mutant did not show negative interaction with mus81 (data not shown) nor hypersensitivity to Zeocin, we think that another function other than Rad53 recruitment (which is lost in the phosphosite mutant) accounts for these phenotypes.

3. A recent paper demonstrated ATR-dependent phosphorylation of the human RecQ helicase WRN. In that context, ATR-dependent phosphorylation would prevent fork inactivation. It seems that association with RPA of Sgs1 could help doing the same job. The authors should evaluate to briefly discuss about similarities and differences between yeast and human RecQ helicases as for what concerns maintenance of stalled fork stability. Indeed, also WRN has …..both at the N and the C-terminal portions of the protein.

We include much more extensive reference to the mammalian homologues both in the introduction, but above all in the discussion.

Referee #2 (Remarks to the Author):

The manuscript "Sgs1-Rpa70 interaction domain stabilizes DNA polymerase a; and recruits Rad53 kinase to stalled forks" by Hegnauer et al describes a separation-of-function allele of Sgs1. The interaction domain between Sgs1 and RPA-70 was mapped where deletion of Sgs1-404-604 reduced

RPA association two-fold. The authors suggest that there may be additional RPA binding sites as well since this reduction was partial and rather weak (especially in the supplemental Figure 2). This region also binds to the FHA domain of Rad53 by yeast-2-hybrid. In this *sgs1*-r1 mutant, polymerase α association to an early origin was epistatic to mec1-100. The authors conclude that RPA-interacting domain is responsible for Sgs1 interaction of Mec1, which would phosphorylate Sgs1 enabling Rad53 recruitment and activation of the DNA damage checkpoint during replication fork arrest caused by HU. The link between Rpa70 interaction with Sgs1 in the same region as DNA polymerase a and the Rad53 kinases not connected well in the manuscript (the EMAP makes the link now, and points to Mus81 and recombinational repair as an R1 domain function). It seems like they are distinct observations. The authors suggest a model in Figure 7B that show a competition between RPA and Rad53 for Sgs1 binding but no data is shown to support this model. (we remove the arrows and suggestion of competition – we have no direct data on this). It would be essential to show data to support this model if it is to be highlighted so prominently in the manuscript. Overall the manuscript adds to our understanding of Sgs1 during DNA replication by pinpointing different regions of Sgs1 that are separate from its helicase and other known domains.

Major comments:

> In Figure 2A, the authors describe testing the Sgs1-B42-AD deletion constructs lacking different sequences based on sequence conservation and conclude that the first two sites are needed for RPA70 binding. It is not clear how these sites are defined and since they very closely overlap with some previously defined acidic regions, it would be helpful to have the conserved sequence alignments shown. It's not obvious why 404-485 and 496-536 are really two distinct sites and not one. This is described in detail in supplemental methods.

2. In Figure 3A, the Sgs1-r1-13MYC appears to be expressed better than the endogenous Sgs1-13MYC and not at comparable WT levels.

The Sgs1-r1-13MYC levels are slightly higher than WT, but not significantly. Previous mutants caused instability of Sgs1, thus slightly more stable is not much of a problem. We do discuss this in the text briefly.

3. In Figure 4, it is a weak argument that Sgs1-RPA interaction contributes to DNA pol a; binding at stalled forks when it was observed for one early origin but not a later firing origin. Additional origins of replication should be analyzed (such as the early origin ARS305 and a corresponding late origin). Multiple origins are tested – both early, mid and late – and are in Figure S4.

4. It is unclear how ITC experiments involving RPA suggest a phosphorylation-independent pathway for interaction between RPA and Sgs1, which is asserted in the discussion in support of Figure 7B.The rationale for these conclusions should be expanded if Figure 7B is to remain part of the manuscript. Sgs1-Rpa Co-IPs were done using S-phase cells (unperturbed), and the ITC binding was done with unmodified peptides. Therefore, the Sgs1-RPA interaction is independent of Sgs1 phosphorylation. In fact, in an earlier paper - Fricke JBC 2001 – there was no Rpa70 interaction with Sgs1, when Sgs1 is phosphorylated (growth on 32P) . We did not see loss of Sgs1-Rpa70 interaction in extracts from cells on HU (Cobb et al., 2003). At least we can say that our data show that unmodified Sgs1 can bind Rpa70.

5. On p. 13 the authors state "We found that Sgs1(404-604) is a very robust substrate for Mec1-Myc phosphorylation, being phosphorylated better than target peptides from Rad53 (Fig. 5C)." It is not clear what target Rad53 peptides are shown in this figure.
PHAS-I, a model PIKK substrate, is a less good substrate, but we now removed this statement as the comparison was not done quantitatively.

Also in Figure 5C, why is the untagged Mec1 not phosphorylating the WT Sgs1-404-604 since this band is not sensitive to caffeine like the tagged version.
The "untagged Mec1" sample does not show modification by Mec1 kinase but shows background due to other kinases, that bind nonspecifically to the beads. Therefore it is not sensitive to caffeine.

6. The competition model shown in Figure 7B between RPA and Rad53 binding is speculative and should not be part of the model shown. It could be discussed as a possibility but there is no evidence for that model in the data shown. Experiments such as an RPA yeast-2-hybrid assay with mutated phosphoacceptor sites on Sgs1 (similar to figure 5C) could be performed, or competition assays with RPA and Rad53 for Sgs1 binding if the authors want to test this hypothesis.
We removed this point; "in vivo" competition is difficult to show and "in vitro" competition may not be relevant.

Minor comments

1. Figure 7 is overly complicated, and could be simplified to highlight the more important parts of the proposed model for the role Sgs1 interactions in replisome stability.
   We removed some unnecessary arrows and hope it is simpler and clearer now. The model does not require nor imply competition.

2. Figure 4 is a difficult figure for the reader, as the figure legend is presented only once and is disjointed. Done

Referee #3 (Remarks to the Author):

In this manuscript, Hegnauer et al. explore the physical interactions of the RecQ helicase Sgs1 with RPA and Rad53, and attempt to determine the significance of these interactions for fork stabilization and checkpoint activation upon replication stress. The authors, and others, have previously shown that:
a. Sgs1 and RPA interact (Cobb Embo J '03)

b. That Sgs1 and Rad53 interact, and have mapped this interaction to the central region of Sgs1, and Rad53's FHA phospho-epitope binding domain. (Bjergbaek Embo J. '05)

c. Sgs1 is important for Rad53 phosphorylation in cells lacking RAD24 (Frei G&D '00)

d. The human homologues of Sgs1 are ATR/ATM targets (Davies, Pichierri, Rao)

In this manuscript, the authors do two things. First (figures 1 & 2 & 5D) they define the interaction domains more precisely, and determine the Kd and stoichiometry of each interaction. This work is well-done and valuable, but doesn't provide much biological insight. Next, the authors show that Sgs1 is a target of Mec1 in vitro, and suggest that Mec1 phosphorylation of Sgs1 promotes the Sgs1-Rad53 interaction, which results in Rad53 recruitment to stalled replication forks. While the previously published data had already led the authors to suggest that Sgs1 recruits Rad53, and thus function in checkpoint activation, this manuscript goes further by suggesting that the recruitment is

mediated directly by Mec1. The problem here is that the authors have not shown that this phosphorylation occurs in vivo, or that it promotes Rad53 binding and recruitment to forks in vivo. Therefore, this paper is not sufficiently novel for publication in EMBO without significant additional experiments to validate the claims made in the last half of the paper.

Extensive substantiation of the second half of the paper is what we now provide.

Suggested experiments:

1. Try to show a phenotype for the *sgs1*-4A mutant. In Figure 6A, the *sgs1*-4A rad24 double mutant does not seem to have a significantly smaller Rad53 shift than the rad24;.
   Rad53 upshift Western blot is improved and time course is shown.
   We tried by combining dpb11-1 with rad24, but this double mutant was too sick on its own to show any additive behavior with *sgs1*-4A or other *sgs1* mutants. We are convinced that combining the *sgs1* mutants with Mrc1-AQ would not help, because Bjerkbaek et al (2005) have clearly shown that for checkpoint function Sgs1 and Mrc1 act on the same pathway.

2. Similarly, in Figure 6B the *sgs1*-4A mutant has no survival defect in long-term growth on HU (not surprising given the result for *sgs1*-r1 in Figure 3E). The *sgs1*-4A mutant should be tested for recovery from acute HU stress as in Figure 4A. Is in supplement, it does not show a recovery defect. This should be done in combination with both rad24
All these experiments were done and are included. However, loss of Rad53 activation is not likely to result in a strong defect in recovery from acute HU stress (cf Berens TJ and Toczyski DP., MBC, 23,1058-1067 (2012) in which it is shown that loss of Rad53 phosphorylation in a *dpb11-1 ddc1* double mutant (or with *tel1* triple) does not affect acute HU survival).

3. Show that Sgs1 is phosphorylated by Mec1 in vivo. Done see explanations above and below.
A. Mass spec on Sgs1 digesting with an enzyme other than trypsin (the authors note that the tryptic fragment is too large). As stated in the text, a combination of Chymotrypsin and Trypsin did not help. We had to use in-gel digest and Chymotrypsin enters the gel pieces even less efficient than Trypsin. Sgs1 could not be efficiently eluted from the beads for an in-solution digest.

B. Showing a Mec1- and damage-dependent gel shift for Sgs1, possibly using Phostag to exaggerate a small shift. Done without Phostag, by using a low concentration (6%) acrylamide gel. We show now a phosphorylation- and Mec1-dependent Sgs1 band shift (Fig 5D,E).

C. IP-ing Sgs1 and then western blotting it with the Cell Signaling anti-S/TQ antibody. Please see comments above. Not necessary

D. Making a phospho-specific antibody to one of the 4 sites mapped on Sgs1. Please see comments above. Not necessary

4. Show that Mec1 phosphorylation of Sgs1 promotes the Sgs1-Rad53 interaction in vivo. The most obvious way to do this is to show that Sgs1-wt and Rad53 co-IP, but sgs1-4A and Rad53 do not. If this is not possible, the relatively modest difference between Sgs1-wt and *sgs1*-4A interaction with Rad53 by yeast two-hybrid (Figure 5E) might be made more convincing by showing that it is dependent on Mec1/Tel1.That is, the authors should repeat both the wt and the 4A in both MEC1 and mec1 strains, and show that mec1 mutants have a lower 2 hybrid, and that this is epistatic to 4A.
We thank the reviewer for this suggestion – we optimized the coIP and now show that *sgs1*-4A

reduces the interaction with Rad53 (Fig 6C). We show quantification from three independent experiments (Fig 6D).

It should be noted that the model that this association is damage driven appears at odds with Fig 2A of the authors 2005 Embo paper, which shows that the Sgs1 Rad53 co-ip is not affected by DNA damage.This needs to be addressed.
This is discussed in the methods section. We see phosphosite dependent Sgs1- Rad53 co-IP but the conditions of IP were not identical to those of Bjergbaek et al. We wash much more stringently (multiple extra washes in 0.5M NaCl, 0.1M Tris) than in the Bjergbaek et al., EMBO J paper.

Major concerns:

5. In Figure 3C, there is significant binding of RPA to beads alone, calling into question the result. Also, the proper negative control is a strain in which Sgs1 is not tagged, not no antibody.
Repeated to reduce binding to beads alone. We detect the 50% reduction in both directions, and there is no background in the reverse direction IP (Fig S2), thus we think this is not a questionable result.

6. Figure 4C and D are used to argue that mec1-100 is epistatic to *sgs1*-r1. However, as in Figure 3E (for which the authors properly note that epistasis cannot be established), there is no convincing defect in the *sgs1*-r1 single mutant. Moreover, the conclusion of the epistasis is not spelled out. If mec1-100 is epistatic, does that mean that it is required for the Sgs1-RPA interaction?
During the recovery experiment suggested by Reviewer 3, we realized that the *sgs1*-r1 cells, which we used in the previous manuscript, carried a mutation that sensitizes cells to acute HU treatment. We backcrossed and analyzed a clean *sgs1*-r1 in acute HU recovery assay. The new result is now shown in Figure 3A. This result led us to conclude that *sgs1*-r1 does NOT impair the fork recovery from HU arrest. We have changed the conclusion that was drawn in the previous manuscript and we appreciate the reviewer's suggestion. This comment, however, is therefore no longer relevant .

Minor concerns:
7. In Figure 3E, the epistasis results might be more clear if mec1 delta; were used in addition to mec1-100. Not relevant
8. In the references in the text to Figure 4, it is suggested that the observed results reflect the disrupted interaction with RPA in the *sgs1*-r1, but they could as easily reflect a disruption of the Sgs1-Rad53 interaction. True – this is changed
9. In the paragraph that begins, "Because HU-treatment...," it should be stated that mec1-100 is epistatic to *sgs1*-r1, not the other way around. Changed
10. Since all tested phenotypes of *sgs1*-r1-hd are similar to *sgs1*-hd, it is unclear what conclusions can be drawn. We include this for the sake of completeness, but we now conclude that the R1 domain does not affect polymerase stabilization after stalling, while the helicase activity is very important.

2nd Editorial Decision 14 June 2012

Thank you for sending in your revised manuscript for our consideration. All three referees have now looked at it once more (see comments below), and I am pleased to inform you that they have no further objections regarding its publication in The EMBO Journal. We shall therefore be happy to accept and publish the study.

For production purposes of the article proper, we shall require individual files both for the text and for EACH of the main figures. Furthermore, to facilitate reader downloads of the supplement, we require all supplementary information (text and figures) to be combined into one single supplementary PDF. I am therefore returning the manuscript to you for an additional minor revision, solely to allow you to easily upload all these required files into our system. You may also want to take this opportunity to briefly comment on referee 3's remaining suggestion, and possibly to incorporate any additional data that you may already have related to the referee's point.

Once we will have received your final manuscript files, we shall be able to swiftly proceed with formal acceptance and production of the paper.

With best regards,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I found the revised ms of Hegnauer and colleagues improved. The authors had positively answered my questions and dealt with my criticisms. Overall, as far as this reviewer can see, authors had also answered the most important concerns raised by the other two reviewers.
In particular, the new experiments included in the revised form of the ms have contributed to improve novelty and make more clear and experimentally-supported statements. Thus, I think that the ms contains several findings that may be of interest to the EMBO Journal readers.

Referee #2 (Remarks to the Author):

Upon re-reading the manuscript, the authors have now addressed all of my specific concerns in the manuscript.

Referee #3 (Remarks to the Author):

The manuscript is improved, most notably in showing that Sgs1 is phosphorylated by Mec1 in vivo. While the shift data look pretty good, I would encourage the authors to also determine whether they can see this with anti-SQ-P antibodies, as currently fig 5D/E are the only data in the paper showing that Sgs1 is actually phosphorylated by mec1. However, I will leave this up to the authors and editor.
The authors have still not actually shown that the Mec1 phosphorylation of Sgs1 is important for recruitment of Rad53, although the defect in Rad53 activation in the single sgs1-r1 or sgs1-4A and the sensitivity of the double rad24 sgs1-r1 to MMS are suggestive.

2nd Revision - authors' response 26 June 2012

You asked us to reply to the comment made by ref. 3, who said that we have not proven that Sgs1 recruits Rad53 upon damage.

True, but it is not possible to CHIP Rad53 so it is not possible to know if they physically interact at damage. On the other hand, functionally the Sgs1 interaction domain is essential for Rad53 activation, and Mec1 phosphorylation is necessary for the interaction, so it is a pretty good bet. We carefully say in the abstract that they interact upon damage response.

We did add some data to Figure 4 - extending the observations of the E-MAP by repeating the assays with specific double mutants. This yielded very interesting results that allowed us to expand our interpretation of the E-MAP data - The figure now bears symbols indicating synthetic lethality under the colored map, and the four genes we confirmed as synthetic lethal (mus81, slx4, slx5 and slx8) are interesting because they are all necessary for resolution of strand exchange figures (cleavage of crossovers). This means that without the sgs1 -R1 domain, these genes become essential. While we already speculated on this with mus81, we now have it with another redundant resolvase activity, strongly reinforcing the functional relevance of the R1, Rpa70 and Rad53 binding domain.

The discussion of this data is on page 12 (second paragraph). Adding this confirmation means that the paper is now slightly over the 55k characters (incl spaces) limit, but I believe that these genetic results are very powerful and therefore should be included. I hope you can accept the slight overrun.

Acceptance letter 28 June 2012

Thank you for submitting your re-revised manuscript for our consideration. I have now had a chance to look through it and to assess your response, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal

