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Assembly of Slx4 signaling complexes behind stalled replication forks

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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.)

Editors: Hartmut Vodermaier

1st Editorial Decision 24 March 2015

Thank you for your patience with our evaluation of your manuscript on Slx4 signaling complexes on replicating DNA. We have now received the comments of all three referees, copied below for your information. As you will see, all referees generally acknowledge the interest and potential importance of this work, yet they also raise a number of critical issues regarding the experimental analyses and their interpretation.

Should you be able to satisfactorily address these concerns in a revised version of the manuscript, we would be happy to consider the study further for publication in The EMBO Journal. However, please keep in mind that we allow only a single round of major revision, making it important to carefully respond to all points raised at this stage. We generally grant three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study; in light of the co-submitted paper it would in the present case nevertheless be desirable to receive a revised version as early as possible, and I would thus be happy to discuss specific outlines/revision plans further if you should consider this helpful.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to your revision.

REFEREE COMMENTS

Referee #1:

In this manuscript, Balint and colleagues report the interesting observation that, in budding yeast, the Slx4 complex assembles behind replication forks in response to replication stress induced by the alkylating agent MMS. They also present evidence that the recruitment of Slx4 depends on the binding of Rtt107 to gamma-H2A(X) and recruits in turn Dpb11 to activate Mec1, the sensor kinase of the DNA damage checkpoint. They propose a model in which Slx4 acts together with Ddc1 (911 complex) to promote the activation of Mec1 at discrete sites in response to MMS treatment. The Smolka lab has recently reported that Slx4 competes with Rad9 for the binding to Dpb11 and reduces the activation of Rad53, the effector kinase of the Mec1-Rad53 pathway (Ohouo et al, 2013). The fact that Slx4 promotes Mec1 activation is therefore very interesting as it indicates that Slx4 acts both as an activator of Mec1 and a repressor of its downstream effector kinase Rad53. This finding is consistent with the fact that Mec1 and Rad53 play distinct roles at stalled replication forks and indicates that Slx4 fine tunes the activity of these two checkpoint kinases, in addition to its role in DNA repair. This represents a very important contribution to the DNA replication and repair field. Overall, the manuscript is well written and the data are of high quality. Yet, the following specific issues need to be addressed prior to publication.

Specific issues:

1) The Slx4 foci shown in Fig. 1A are not very convincing. They do not really look like subnuclear foci and the fact that there is no staining of the nucleus does not help. The intensity of the GFP signal seems to be stronger in the MMS-treated cells but since less than 20% of cells show this pattern after two hours of MMS exposure, it is not clear what it corresponds to. To support their statement, the authors should follow the kinetics of foci formation over time and compare it to the fraction of cells in S phase. They should also compare the kinetics of Slx4 foci formation to the kinetics of other types of foci, such as Rad52 or Ddc1. Alternatively, they could remove this panel as it does not bring much to the story.

2) The ChIP-seq data are nice and convincing. These data show a clear enrichment of Slx4 in the vicinity of early origins. The use of input DNA to identify replicated regions as CNVs is also very smart. However, the authors should provide some positive control to show that the CNV profile gives similar results than a BrdU or EdU profiles. For instance, they could compare the spreading of Dpb3 signals (Fig. 1F) with CNVs in the same samples. Finally, it is not clear why the authors have labeled Fig. 1C as "Slx4 CNV". As I understand, this panel corresponds to CNVs in wt cells exposed to MMS, so what does Slx4 mean?

3) Fig 1E shows that SLX4 is enriched at early origins relative to late origins. The authors should use a statistical test to confirm that this difference is significant (also true for Fig. 2G, 3D, 4C). It would also be interesting to correlate Slx4 enrichment with the time of origin activation (Trep).

4) The authors conclude from Figure 1 that Slx4 accumulates behind replication forks in MMS-treated cells (page 5, first paragraph), in a region that is spatially distinct from that occupied by the replication. An alternative possibility could be that SLX4 accumulates at stalled or damaged forks. Since these forks lag behind the bulk of active forks, this would explain why the signals from damaged and intact forks are spatially distinct.

5) The panel showing that the DNA copy number profile is the same in wt and rtt107 cells (Fig. E1A) should be shown in Fig. 2.

6) In the second paragraph of page 7, the authors discuss the fact that Dpb11 enrichment looks like Rtt107 and Slx4, but with lower amplitude. This is not obvious from the figures, especially if one considers that it is difficult to compare the amplitude of ChIP signals from different proteins.

7) In the first sentence of the Discussion section, the authors stress the fact that "... Slx4 protein complexes assemble in response to replication stress, ...". However, they have investigated Slx4 recruitment to replicated sites only in the presence of MMS. It would be important to determine

whether Slx4 also bind replicated regions in the presence of HU, which does not generate DNA lesions. Moreover, the authors should look at Slx4 recruitment in unchallenged growth conditions, to confirm that this recruitment is due to replication stress.

8) In the same paragraph, the authors conclude that Slx4 forms a "H2A-Ser129-P/Rtt107/Slx4/Dpb11 multiprotein complex". This is a very likely possibility but is not directly shown in the manuscript, so this statement should be toned down.

9) Along the same line, the statement that "Mec1 is active at the same chromosomal sites where Slx4 is recruited, since Slx4 and H2A-Ser129-P co-localize extensively" (page 12, second paragraph) is not supported by the data shown Fig.5D. Indeed, the fact that the two average profiles look similar does not necessarily mean that the intensity of individual signals correlates. To support this statement, the authors should plot the intensity of individual Slx4 and H2A-Ser129-P signals and calculate the corresponding correlation coefficient.

10) Page 12 (bottom), the authors propose two possible mechanisms by which Slx4 could stimulate Mec1 activity. It would help to draw a model to illustrate these two possibilities.

Referee #2:

This is an interesting study where the authors combine ChIP seq, standard biochemistry, genetics and DNA fiber analysis to study the timing of recruitment of Slx4, Rtt107 and Dpb11 to chromatin during replication stress. The results are overall of good quality and the paper is nicely crafted with an interesting discussion that tames down what is felt like over-interpretations of some of the data in the Results section.
Overall the findings made using a genome wide analysis approach are overall in agreement with the co-submitted manuscript by Cussiol et al. and both papers are quite complementary of one another.
Although I make some comments and raise some concerns regarding various aspects of this study, I believe that it should be of interest to the readers on the EMBO journal. It has the merit of providing a global view on the role played by Slx4 and Rtt107 in response to replication stress and in the dampening of the Rad53 mediated checkpoint response at stalled/stressed replication forks while promoting Mec1 signaling. However, as explained below this global approach can in some cases be a source of concern and the authors really need to take precautions in the interpretation of their data to avoid some approximations.

General Comment:
The authors throughout describe Slx4 and Rtt107 as being recruited "behind" the fork. What does "behind" really mean. What distances are we talking about? We are taking here a macroscopic view on things. The data clearly show that there is a gradient of Slx4 and Rtt107 on chromatin, with amounts increasing as you move away from the replication fork back toward the origin.
Slx4 is found in considerably lower amounts at regions positive for Dpb3 (Pol epsilon) compared to those found further away as we move back towards the origin. This is an important observation that strongly supports the idea that Slx4 is not part of the replisome.
However, this does not mean that some Slx4 could be recruited to the fork where it could fulfill key functions. How do we know that those relatively low amounts of Slx4 at the fork are not those that are important and that the excess of Slx4-Rtt107 that accumulates further away from the fork does not "just" result from the amplification of Mec1 signaling that it has itself initiated at or near the fork by replacing Rad9? Are we not reaching the detection limits of the method?

Along those lines, the authors propose in their discussion that "Slx4 functions in concert with Ddc1 to recruit the Mec1 activator Dpb11 during the replication stress response".
Their ChIP seq data showing that Dpb11 needs Slx4 and Rtt107 to be recruited do support this idea. However, in their co-submitted manuscript by Cussiol and colleagues, the authors propose a model where Dpb11 is already there and where the recruitment of Slx4-Rtt107 is necessary to replace Rad9 within the pre-existing Ddc1-Dpb11-Rad9-H2AS129P complex at the stalled/stressed fork rather than to recruit Dpb11. This would then result in both a relief of Rad53 activation and the amplification spiral of Mec1 signaling which will promote phosphorylation of Slx4, Rtt107 and

H2A. This subsequently leads to more recruitment of Dpb11 and further Mec1 activation etc... All of which may explain the strong accumulation of Slx4, Rtt107 and Dpb11 seen by ChIP seq as we move upstream away from the fork.
Would it not be best to present things in this manuscript too with a two step model where
1- Slx4-Rtt107 replace Rad9 at or just behind the stressed fork dampening Rad53 activity while promoting Mec1 signaling
2- This is followed by a secondary wave of recruitment of Slx4-Rtt107-Dpb11 via interaction of Rtt107 with H2AS129P, which will further amplify Mec1 signaling etc... and lead to levels that become detectable by ChIP seq further away from the fork.

The first initiating event may be triggered by the recruitment of Slx4 at, or not far from, the stressed fork. The initially low levels of Slx4-Rtt107 and the distance from the stressed fork may not be detectable by the macroscopic view provided by ChIP seq data (relative to a stressed replication fork and to levels of protein on chromatin). The levels of Slx4-Rtt107-Dpb11 that can be detected by ChIP seq would be seen only further away "behind the fork" after further amplification of Mec1 signaling and further phosphorylation of H2A.
The authors do nicely discuss the idea, in relation with the recent Gritenaite et al 2014 paper and their own findings in the co-submitted Cussol et al. paper, that the accumulation of Slx4-Rtt107-Dpb11 away from the fork could be important to promote the resolution of recombination intermediates after the recruitment of replacement of Slx4 Mus81-Mms4 via binding of phosphorylated Mms4 with Dpb11.

Specific comments per figure:

Figure 1:
Q1 What is the distribution of SLX4 when released in absence of MMS?
Q2 What is the distribution of SLX4 when MMS is added later after release of the G1 synchronized cells?
Q3 What does the distribution of SLX4 look like at 0 min?
Q4 It would help the interpretation of the data if graphs were provided with superimposed kinetics curves of Dpb3 and Slx4 distribution (maybe as Supp data).

Figure 2:
Q5 What are the precise boundaries of the Slx4-1, 2, 3, 4 and 5 fragments used in Y2H for mapping the Rtt107 binding region (Figure E1C).

Figure 3
Q6 Loss of H2A phosphorylation does not abolish the recruitment of Rtt107 at early origins. It looks like Rtt107 still gets recruited but that the peaks are much narrower and not as high than in WT conditions, but the peaks at or around the early origins are definitely still there. What really seems to change is that many new peaks appear in regions that were totally devoid of Rtt107 in the WT H2A strain. For example, a high narrow peak comes up in the h2a-s129a mutant around 0.03 Mbp. Another striking example is around position 0.3 Mbp where plenty of small peaks show up in the h2a-s129a mutant. Those regions contain late origins. Does preventing H2A phosphorylation have any consequence on firing of late origins? Is Rtt107 getting recruited at or around late origins? The quantitative data in Figure 3D certainly seem to be going that way. This is not at all discussed in the manuscript. How is it getting recruited to chromatin if there is no more phosphorylated H2A to bind too?

Q7 Related to the above question would it not be informative to compare the profile of Rtt107 and Dpb3 in the h2as129a strain to see whether the new peaks of Rtt107 are related to replicated regions?

Q8 What is the dip in the peak of RTT107 in h2as129a?

Figure 4

The data show two apparently independent patterns of recruitment of Dpb11 at early origins. One results in a narrow and sharp peak right at the origin, while the second one results in a broad peak centered around the origin.
The data convincingly show that the latter pattern is dependent on Slx4, Rtt107 and Ddc1 while the former one isn't at all.

Q9 What time after release in MMS are we looking at? It would have been nice to have a kinetic analysis of the recruitment of Dpb11 along with that of Slx4 (or Rtt107), Ddc1 and Dpb3 (Pol epsilon) to get an idea of the chronology of events.

Figure 6
Q10 Why don't the authors comment on the fact that the slx4-s486a mutant looks just as impaired in MMS recovery than the slx4-delta mutant in Fig 6B?

Referee #3:

In this manuscript Balint and coworkers investigate the role of Slx4 following MMS-induced replication stress.
Slx4 is a very intriguing protein, which seems to have multiple roles, none of which is fully understood. The authors contribute to our understanding of Slx4 by providing a large set of very clean data that adds some relevant information to the puzzle.
The authors propose that Slx4 is recruited behind stressed replication forks through its interaction with Rtt107, which binds to Mec1-phosphorylated H2A. Recruitment of Slx4 leads to its phosphorylation and triggers a competition for binding to Dpb11 between Slx4 and Rad9. Formation of a Rtt107-Slx4-Dpb11 complex has at least two effects: on one hand it promotes Mec1 activation in a positive feedback loops that result in Rtt107 phosphorylation, on the other hand, by interfering with Rad9-Dpb11 interaction, it dampens activation of Rad53. Thus Slx4, according to the model, has opposite effects on the two checkpoint kinases, possibly suggesting different roles for Mec1 and Rad53 in responding locally and globally, respectively, to genotoxic stress. This is a very interesting concept that may help understand some of the grey area in the field.
The authors performed a huge amount of work to obtain convincing and well controlled results; the data presented are fully consistent with the model.
I think that this is an elegant work that adds another bit of information regarding how cells manage in the presence of replication stress.
I have only a couple minor comments:
- The authors suggest that Dpb11 recruitment is partially due to Slx4 and partly to Ddc1 interaction, indeed they partially lose Dpb1 binding to chromatin both in slx4Δ and in ddc1Δ. I might have missed something, but what happens in a ddc1Δslx4Δ strain? According to the model the expectation would be to lose completely Dpb11 binding. On the other hand, if Ddc1 and Slx4 cooperated for the recruitment of Dpb11 through the same mechanism, the double mutant may reveal an independent mechanism for Dpb11 loading.
- In figure 4 the author report the binding distribution of Dpb11 around early firing ARSs in replication stress conditions. It would be interesting to compare these results with Dpb11 binding in unstressed cells entering S phase.

1st Revision – authors' response 06 May 2015

Response to reviewers.

Thanks to the reviewers for their insightful comments, and to the editor for providing guidance for our response. The comments of each reviewer are restated below, followed by our response:

Referee #1:

In this manuscript, Balint and colleagues report the interesting observation that,

in budding yeast, the Slx4 complex assembles behind replication forks in response to replication stress induced by the alkylating agent MMS. They also present evidence that the recruitment of Slx4 depends on the binding of Rtt107 to gamma-H2A(X) and recruits in turn Dpb11 to activate Mec1, the sensor kinase of the DNA damage checkpoint. They propose a model in which Slx4 acts together with Ddc1 (911 complex) to promote the activation of Mec1 at discrete sites in response to MMS treatment. The Smolka lab has recently reported that Slx4 competes with Rad9 for the binding to Dpb11 and reduces the activation of Rad53, the effector kinase of the Mec1-Rad53 pathway (Ohouo et al, 2013). The fact that Slx4 promotes Mec1 activation is therefore very interesting as it indicates that Slx4 acts both as an activator of Mec1 and a repressor of its downstream effector kinase Rad53. This finding is consistent with the fact that Mec1 and Rad53 play distinct roles at stalled replication forks and indicates that Slx4 fine tunes the activity of these two checkpoint kinases, in addition to its role in DNA repair. This represents a very important contribution to the DNA replication and repair field. Overall, the manuscript is well written and the data are of high quality. Yet, the following specific issues need to be addressed prior to publication.

Specific issues:

1) The Slx4 foci shown in Fig. 1A are not very convincing. They do not really look like subnuclear foci and the fact that there is no staining of the nucleus does not help. The intensity of the GFP signal seems to be stronger in the MMS-treated cells but since less than 20% of cells show this pattern after two hours of MMS exposure, it is not clear what it corresponds to. To support their statement, the authors should follow the kinetics of foci formation over time and compare it to the fraction of cells in S phase. They should also compare the kinetics of Slx4 foci formation to the kinetics of other types of foci, such as Rad52 or Ddc1. Alternatively, they could remove this panel as it does not bring much to the story.

We have carried out a more complete analysis of Slx4 nuclear foci, now shown in Figure 1A-D. We included Nup49-RFP to mark the nuclei, and quantified Slx4 focus formation in asynchronous culture with and without MMS, and in cultures proceeding synchronously through S phase. Slx4 foci are surprisingly abundant during unperturbed S phase, and increase in the presence of MMS, both in terms of cells with foci and foci per cell. In the presence of MMS almost 100% of S phase cells have Slx4 foci. Our data indicate that Slx4 foci form mostly in S phase, as has been reported in the literature for Rad52 foci.

2) The ChIP-seq data are nice and convincing. These data show a clear enrichment of Slx4 in the vicinity of early origins. The use of input DNA to identify replicated regions as CNVs is also very smart. However, the authors should provide some positive control to show that the CNV profile gives similar results than a BrdU or EdU profiles. For instance, they could compare the spreading of Dpb3 signals (Fig. 1F) with CNVs in the same samples. Finally, it is not clear why the authors have labeled Fig. 1C as "Slx4 CNV". As I understand, this panel corresponds to CNVs in wt cells exposed to MMS, so what does Slx4 mean?

As requested, we have overlaid the CNV on the Dpb3 ChIP signal for all three time points, and now show this as Figure E1C and include a comment on p.5. We have re-labeled Figure 1C (now Figure 1F) to simply read “CNV” (we previously used Slx4 CNV simply to indicate that the CNV was from the same sample as the Slx4 ChIP, but this is clear in the figure legend).

3) Fig 1E shows that SLX4 is enriched at early origins relative to late origins. The authors should use a statistical test to confirm that this difference is significant (also true for Fig. 2G, 3D, 4C). It would also be interesting to correlate Slx4 enrichment with the time of origin activation (Trep).

We have added p-values from Wilcoxon rank sum tests to all of the boxplots. We have compared median Slx4 enrichment score for all origins to the Trep data from Yabuki et al, as curated in oriDB, and find a negative correlation, indicating that origins that replicate earlier (small Trep) have increased Slx4 enrichment. The correlation plot is now shown in Figure E1A, and a comment in the text, on p.5, has been added.

4) The authors conclude from Figure 1 that Slx4 accumulates behind replication forks in MMS-treated cells (page 5, first paragraph), in a region that is spatially distinct from that occupied by the replication. An alternative possibility could be that SLX4 accumulates at stalled or damaged forks. Since these forks lag behind the bulk of active forks, this would explain why the signals from damaged and intact forks are spatially distinct.

This is a formal possibility that we can't exclude. It is predicated on there being some small amount of stalled forks in MMS that accounts for all of the Slx4 signal. The DNA combing did not reveal a subpopulation of tracks of small size that might indicate that they lag behind the bulk, but if they exist they could be below the detection limit. A small peak of Dpb3 remains at the origin even at 90 minutes, but it is not known if this represents stalled forks. We now discuss this possibility on p.11.

5) The panel showing that the DNA copy number profile is the same in wt and rtt107 cells (Fig. E1A) should be shown in Fig. 2.

We have moved E1A to Fig. 2.

6) In the second paragraph of page 7, the authors discuss the fact that Dpb11 enrichment looks like Rtt107 and Slx4, but with lower amplitude. This is not obvious from the figures, especially if one considers that it is difficult to compare the amplitude of ChIP signals from different proteins.

We removed the reference to amplitude on p.7.

7) In the first sentence of the Discussion section, the authors stress the fact that "... Slx4 protein complexes assemble in response to replication stress, ...". However, they have investigated Slx4 recruitment to replicated sites only in the presence of MMS. It would be important to determine whether Slx4 also bind replicated regions in the presence of HU, which does not generate DNA lesions. Moreover, the authors should look at Slx4 recruitment in unchallenged growth conditions, to confirm that this recruitment is due to replication stress.

The reviewer is correct that we have performed ChIP only in the presence of MMS. Our improvements to the Slx4 foci analysis (Figure 1B) helps with this point, as it shows more Slx4 foci per cell in MMS. We have changed the sentence noted to 'in response to MMS induced DNA replication stress', to clarify the statement. There are a few points that address the broader issue, which is whether Slx4 complexes assemble behind unperturbed forks. First, the improved Slx4 foci analysis (Figure 1B) shows that more Slx4 foci form per cell when MMS is present (although we would not argue that an Slx4 focus is necessarily equivalent to an Rtt107-Slx4-Dpb11 complex. It is established in the literature that Dpb11 does not travel with the unperturbed replication fork (PMID: 10733584), reinforcing our inference that Slx4 complexes assemble during replication stress. Our inference that Slx4 complexes assemble is further supported by evidence that the three phosphorylation events that are required for assembly (P-H2A, P-Slx4, and P-Ddc1) are all greatly increased in replication stress. We also show that the abundance of Slx4 complexes increases over time in MMS, further suggesting that Slx4 complexes assemble during stress. We added a more complete discussion on p.11 to outline the evidence suggesting that the complexes assemble in stress. We also acknowledge that similar

assembly could occur in an unperturbed S phase, at the fraction of forks that experience stress without external perturbation.
The unperturbed ChIP is a very challenging experiment due to fork asynchrony and fork speed. We agree that it would be interesting to probe the status of Slx4 complexes during an unperturbed S phase, but note that our evidence that Slx4 complexes are present during MMS induced replication stress is not invalidated by either the presence or absence of Slx4 complexes during unperturbed S.
We have not analyzed Slx4 recruitment in HU, because *slx4Δ* is not HU sensitive, and modes of recruitment could differ in different agents. Our preference is to focus on a single agent.

8) In the same paragraph, the authors conclude that Slx4 forms a "H2A-Ser129-P/Rtt107/Slx4/Dpb11 multiprotein complex". This is a very likely possibility but is not directly shown in the manuscript, so this statement should be toned down.

We changed the statement on p.11 to “Slx4 likely forms” to tone it down. The details of the individual interactions that lead to our conclusion are detailed and referenced in the rest of the paragraph, and, as the reviewer points out, are not shown by us in the manuscript.

9) Along the same line, the statement that "Mec1 is active at the same chromosomal sites where Slx4 is recruited, since Slx4 and H2A-Ser129-P colocalize extensively" (page 12, second paragraph) is not supported by the data shown Fig.5D. Indeed, the fact that the two average profiles look similar does not necessarily mean that the intensity of individual signals correlates. To support this statement, the authors should plot the intensity of individual Slx4 and H2ASer129-P signals and calculate the corresponding correlation coefficient.

We conducted the analysis suggested by the reviewer, comparing Slx4 to P-H2A, Slx4 to Dpb3 (which we expect to be poorly correlated), Slx4 to Rtt107 (which we expect to be highly correlated), and Slx4 to an independent replicate of Slx4. We compared enrichment scores across 50kb upstream and downstream of the 108 early firing origins, to compare the profiles shown in Figure 5D. Strong positive correlations were evident when Slx4 was compared to Slx4, to Rtt107, and to H2A-Ser129-P. The correlation with Dpb3 was lesser, and the x-y plot reveals clearly that the correlation of Slx4 with Dpb3 is not accurately reflected as a linear relationship. These data are presented on p.9 and in Figure E3.

10) Page 12 (bottom), the authors propose two possible mechanisms by which Slx4 could stimulate Mec1 activity. It would help to draw a model to illustrate these two possibilities.

We added a model figure (Figure 7) to clarify these mechanisms, and to summarize our findings.

Referee #2:

This is an interesting study where the authors combine ChIP seq, standard biochemistry, genetics and DNA fiber analysis to study the timing of recruitment of Slx4, Rtt107 and Dpb11 to chromatin during replication stress. The results are overall of good quality and the paper is nicely crafted with an interesting discussion that tames down what is felt like over-interpretations of some of the data in the Results section.
Overall the findings made using a genome wide analysis approach are overall in agreement with the co-submitted manuscript by Cussiol et al. and both papers are quite complementary of one another.
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Rad53 mediated checkpoint response at stalled/stressed replication forks while promoting Mec1 signaling. However, as explained below this global approach can in some cases be a source of concern and the authors really need to take precautions in the interpretation of their data to avoid some approximations.
General Comment:
The authors throughout describe Slx4 and Rtt107 as being recruited "behind" the fork. What does "behind" really mean. What distances are we talking about? We are taking here a macroscopic view on things. The data clearly show that there is a gradient of Slx4 and Rtt107 on chromatin, with amounts increasing as you move away from the replication fork back toward the origin.
Slx4 is found in considerably lower amounts at regions positive for Dpb3 (Pol epsilon) compared to those found further away as we move back towards the origin. This is an important observation that strongly supports the idea that Slx4 is not part of the replisome.
However, this does not mean that some Slx4 could be recruited to the fork where it could fulfill key functions. How do we know that those relatively low amounts of Slx4 at the fork are not those that are important and that the excess of Slx4-Rtt107 that accumulates further away from the fork does not "just" result from the amplification of Mec1 signaling that it has itself initiated at or near the fork by replacing Rad9? Are we not reaching the detection limits of the method?

We have toned down 'behind' in the Results to reflect the gradient suggested by the reviewer, and make a more balanced discussion on p.12. If, as the reviewer points out, the data strongly suggest that Slx4 is not part of the replisome, then it must be distal to the replisome. Slx4 is clearly not in front of the replisome in any of our experiments, therefore we describe Slx4 as accumulating behind the replisome. I agree that claims as to how far behind the replisome Slx4 accumulates, or as to whether there might be sub-domains within the Slx4 domain would be beyond the resolution of the method and the limitations of the population view. We discuss the possibility of a transition to Mus81/Mms4 complexes, and have added some discussion of a possible transition from Rad9 complexes (see below).
The model the reviewer suggests, where a small amount of Slx4 at the replisome provides key functions, whereas the detectable Slx4 distal to the fork is not biologically functional, while conceivable, is not well supported by the available data. First, it seems unnecessarily complicated to invoke special function of a small or possibly even non-existent sub-population of Slx4 (only a very small amount of Slx4 recruitment is evident at 30 minutes, a time when early origins are robustly engaged by Dpb3; Figure 1I). Second, combing analysis presented in the manuscript, and previously by the Pasero lab, does not detect alterations in fork kinetics, alterations that might be expected if fork-proximal Slx4 was executing a key function there. Third, our evidence and that from the Smolka and Russell labs suggest that P-H2A is largely responsible for recruitment of Rtt107-Slx4, and it is unlikely that normal nucleosome density is present directly at the replisome, where ssDNA predominates. Fourth, if a population of Slx4 was replisome-associated, we would expect a hybrid pattern in the Slx4 ChIP timecourse, with a peak coincident with Dpb3 in addition to the fork-distal peak.
Perhaps the comment is directed more at the possibility that fork-proximal Slx4 is more functional than fork-distal Slx4? As the reviewer points out, the methodology is likely not up to the task of such high-resolution dissection. However, there is good agreement with the location of the bulk Slx4 with the location of Dpb11. Since Dpb11 recruitment is the key function of Slx4 that we propose, it appears reasonable to infer that this function is carried out by the forkdistal Slx4 that we detect.
The population aspects inherent in ChIP certainly have limitations, but the method has the unique ability to detect protein association with chromatin regions in vivo, and provides a view that is not afforded by analysis of protein-protein interactions in vitro or in cell extracts.

Along those lines, the authors propose in their discussion that "Slx4 functions in

concert with Ddc1 to recruit the Mec1 activator Dpb11 during the replication stress response".
Their ChIP seq data showing that Dpb11 needs Slx4 and Rtt107 to be recruited do support this idea.

We agree that the ChIP seq data support this idea (as does our data that Slx4 recruitment does not require Dpb11 interaction). That stable binding of Dpb11 required both Slx4 and Ddc1 fits nicely with the two-site docking model of Dpb11 engagement proposed in the Cussiol manuscript.

However, in their co-submitted manuscript by Cussiol and colleagues, the authors propose a model where Dpb11 is already there and where the recruitment of Slx4-Rtt107 is necessary to replace Rad9 within the pre-existing Ddc1-Dpb11-Rad9-H2AS129P complex at the stalled/stressed fork rather than to recruit Dpb11. This would then result in both a relief of Rad53 activation and the amplification spiral of Mec1 signaling which will promote phosphorylation of Slx4, Rtt107 and H2A. This subsequently leads to more recruitment of Dpb11 and further Mec1 activation etc... All of which may explain the strong accumulation of Slx4, Rtt107 and Dpb11 seen by ChIP seq as we move upstream away from the fork.

In the context of our manuscript we have been unable to find clear evidence of a pre-existing Ddc1-Dpb11-Rad9-PH2A complex on chromatin, for a variety of possibly technical reasons. In particular, Rad9 does not ChIP effectively in MMS in our hands. In Cussiol et al, there is evidence of such a complex in the co-IP analysis, making presentation of the transition model more appropriate in their manuscript. We agree that amplification of Mec1 signaling could promote such a transition, and have added some discussion indicating that the Mec1 amplification cycle could be a means of reinforcing the transition from Rad9 to Slx4 complexes, on p.15. The nature of the transition on chromatin remains uncertain at this point. We feel that a model where a key competition point is PH2A (i.e., Rtt107 displaces Rad9 from P-H2A, releasing Dpb11 that can reassociate by engaging Slx4 and Ddc1), rather than Dpb11, is also consistent with the data, and in particular is supported by the large decrease in Dpb11 occupancy when either Ddc1 or Slx4 is absent and the total independence of Slx4 recruitment from Dpb11 binding that we observe. We now discuss Rad9 transition models on pp.12-13.

Would it not be best to present things in this manuscript too with a two step model where
1- Slx4-Rtt107 replace Rad9 at or just behind the stressed fork dampening Rad53 activity while promoting Mec1 signaling
2- This is followed by a secondary wave of recruitment of Slx4-Rtt107-Dpb11 via interaction of Rtt107 with H2AS129P, which will further amplify Mec1 signaling etc... and lead to levels that become detectable by ChIP seq further away from the fork.

We have incorporated some aspects of a transition model in the discussion on pp. 12-13, with the caveat that the nature of the Rad9 to Slx4 transition is unclear mechanistically. We agree that there is good support for step 2, but for me step 1 is too speculative at this point. The status of Rad9 on chromatin is not clear, and evidence that Dpb11 is recruited independently of Slx4 in detectable amounts is currently lacking.

The first initiating event may be triggered by the recruitment of Slx4 at, or not far from, the stressed fork. The initially low levels of Slx4-Rtt107 and the distance from the stressed fork may not be detectable by the macroscopic view provided by ChIP seq data (relative to a stressed replication fork and to levels of protein on chromatin). The levels of Slx4-Rtt107-Dpb11 that can be detected by ChIP seq would be seen only further away "behind the fork" after further amplification of

Mec1 signaling and further phosphorylation of H2A.

It is certainly possible that Slx4 on chromatin is below detection levels near to forks at early time points, but unless a more complex 'masking' model is invoked the bottom line is that we readily detect Slx4 complexes more distal to the fork.

The authors do nicely discuss the idea, in relation with the recent Gritenaite et al 2014 paper and their own findings in the co-submitted Cussol et al. paper, that the accumulation of Slx4-Rtt107-Dpb11 away from the fork could be important to promote the resolution of recombination intermediates after the recruitment of replacement of Slx4 Mus81-Mms4 via binding of phosphorylated Mms4 with Dpb11.
Specific comments per figure:
Figure 1:
Q1 What is the distribution of SLX4 when released in absence of MMS?
Q2 What is the distribution of SLX4 when MMS is added later after release of the G1 synchronized cells?
Q3 What does the distribution of SLX4 look like at 0 min?

Q1 – Q3 are all variations of ChIP in unperturbed S phase, and are discussed in our response to Reviewer 1. We show the distribution of Slx4 at 30 minutes in Figure 1I, and find there is little Slx4 binding. We would expect even less at 0 minutes.

Q4 It would help the interpretation of the data if graphs were provided with superimposed kinetics curves of Dpb3 and Slx4 distribution (maybe as Supp data).

We have added these to Figure E1.

Figure 2:
Q5 What are the precise boundaries of the Slx4-1, 2, 3, 4 and 5 fragments used in Y2H for mapping the Rtt107 binding region (Figure E1C).

We have added the boundaries to what is now Figure E2B.

Figure 3
Q6 Loss of H2A phosphorylation does not abolish the recruitment of Rtt107 at early origins. It looks like Rtt107 still gets recruited but that the peaks are much narrower and not as high than in WT conditions, but the peaks at or around the early origins are definitely still there. What really seems to change is that many new peaks appear in regions that were totally devoid of Rtt107 in the WT H2A strain. For example, a high narrow peak comes up in the h2a-s129a mutant around 0.03 Mbp. Another striking example is around position 0.3 Mbp where plenty of small peaks show up in the h2a-s129a mutant. Those regions contain late origins. Does preventing H2A phosphorylation have any consequence on firing of late origins? Is Rtt107 getting recruited at or around late origins? The quantitative data in Figure 3D certainly seem to be going that way. This is not at all discussed in the manuscript. How is it getting recruited to chromatin if there is no more phosphorylated H2A to bind too?

In the S129A mutant the Rtt107 peaks are significantly reduced but not eliminated. The reduction is evident in the quantification in Figure 3F and we now show the early ARS averages in Figure 3D. The reduction in Rtt107 binding varies depending on the specific origin, for reasons that we do not understand. For example, binding at the ARS at 0.68Mbp and at 0.2 Mbp is more clearly reduced than at the ARS at 0.37 Mbp. In addition, Rtt107 binding at many coordinates is evident, suggesting to us an increase in non-specific binding. While many of these regions contain late origins, the Rtt107 peaks seem to only randomly associate with late origins vs non-origins. Analysis of the CNV profiles

shows at most a small copy number increase at late origins in the average view of S129A (shown below). We agree that the data suggest that in addition to the H2A-P mode of recruitment there must be an additional mode of Rtt107 recruitment, but the data indicate that the additional mode is a minor one in MMS. Importantly, deletion of *RTT107* eliminates Slx4 binding and so irrespective of the recruitment mode, Rtt107 is necessary for Slx4 complex assembly. We clarify that Rtt107 recruitment is reduced but not eliminated in S129A, on p.7. Late origin firing appears to be modest, and could be consistent with checkpoint signaling defects, but is peripheral to the focus of the manuscript.

Q7 Related to the above question would it not be informative to compare the profile of Rtt107 and Dpb3 in the h2as129a strain to see whether the new peaks of Rtt107 are related to replicated regions?

As noted above, replication can be assessed by analysis of CNV, and did not reveal significant late origin firing.

Q8 What is the dip in the peak of RTT107 in h2as129a?

The dip in the peaks in Figure 3E, which is a CNV profile, is likely due to differences between the G1 control genomic DNA sample, and the experimental strain. Different strains often have different 'loads' of Ty elements. Some of these are within origins, but because of their repetitive nature Ty elements map nonspecifically (so when the sequencing reads are mapped back to the reference genome, Ty sequences map to all Ty elements). This can give the appearance that there is less DNA at the 'average' origin than the regions immediately flanking it, which of course is highly unlikely. We infer instead that our G1 control DNA strain had more Ty copies than either of the RTT107-flag strains used in the experiment, a likely possibility given that the G1 DNA is from an S288C strain whereas the S129A strains are W303.

Figure 4
The data show two apparently independent patterns of recruitment of Dpb11 at early origins. One results in a narrow and sharp peak right at the origin, while the second one results in a broad peak centered around the origin.
The data convincingly show that the latter pattern is dependent on Slx4, Rtt107 and Ddc1 while the former one isn't at all.
Q9 What time after release in MMS are we looking at? It would have been nice to have a kinetic analysis of the recruitment of Dpb11 along with that of Slx4 (or Rtt107), Ddc1 and Dpb3 (Pol epsilon) to get an idea of the chronology of events.

This experiment was done at the standard 60 minutes after release into MMS. We have not performed time courses with Dpb11, but it is worth keeping in mind that at the earlier time point, 30 minutes, we show that Dpb3 is engaged in a single sharp origin-proximal peak. Thus it is difficult to assess Dpb11 recruitment to the regions that depend on Slx4 and Ddc1 as the forks will not have reached these regions at the earlier time point. We see no change in the sharp origin proximal peak of Dpb11 upon deletion of RAD9, and so we have no expectation that the earlier time point will reveal the putative Rad9-dependent recruitment of Dpb11 as it would be obscured by the Rad9/Slx4/Ddc1 independent Dpb11 peak.

Figure 6
Q10 Why don't the authors comment on the fact that the slx4-s486a mutant looks just as impaired in MMS recovery than the slx4-delta mutant in Fig 6B?

We agree that the flow cytometry assay does not resolve a difference between *S486A* and *slx4Δ*, whereas the MMS sensitivity assay does. We now indicate that *slx4-s486a* recovers slowly from MMS, like *slx4Δ* and *slx4-bd*, on p.10.

Referee #3:

In this manuscript Balint and coworkers investigate the role of Slx4 following MMS-induced replication stress.
Slx4 is a very intriguing protein, which seems to have multiple roles, none of which is fully understood. The authors contribute to our understanding of Slx4 by providing a large set of very clean data that adds some relevant information to the puzzle.
The authors propose that Slx4 is recruited behind stressed replication forks through its interaction with Rtt107, which binds to Mec1-phosphorylated H2A. Recruitment of Slx4 leads to its phosphorylation and triggers a competition for binding to Dpb11 between Slx4 and Rad9. Formation of a Rtt107-Slx4-Dpb11 complex has at least two effects: on one hand it promotes Mec1 activation in a positive feedback loops that result in Rtt107 phosphorylation, on the other hand, by interfering with Rad9-Dpb11 interaction, it dampens activation of Rad53. Thus Slx4, according to the model, has opposite effects on the two checkpoint kinases, possibly suggesting different roles for Mec1 and Rad53 in responding locally and globally, respectively, to genotoxic stress. This is a very interesting concept that may help understand some of the grey area in the field.
The authors performed a huge amount of work to obtain convincing and well controlled results; the data presented are fully consistent with the model.
I think that this is an elegant work that adds another bit of information regarding how cells manage in the presence of replication stress.
I have only a couple minor comments:
- The authors suggest that Dpb11 recruitment is partially due to Slx4 and partly to Ddc1 interaction, indeed they partially lose Dpb1 binding to chromatin both in slx4Δ and in ddc1Δ. I might have missed something, but what happens in a ddc1Δslx4Δ strain? According to the model the expectation would be to lose completely Dpb11 binding. On the other hand, if Ddc1 and Slx4 cooperated for the recruitment of Dpb11 through the same mechanism, the double mutant may reveal an independent mechanism for Dpb11 loading.

Our inference from Figure 4A and 4D is that Dpb11 binding to the origin distal regions (the broad part of the peak) is completely lost (returns to the baseline) in both *slx4Δ* and *ddc1Δ*. Therefore we didn't assess a double mutant as the assay has no remaining dynamic range of Dpb11 binding to detect. It is possible that the sharp origin proximal portion of the peak, which does not depend on *SLX4* or *DDC1* or *RAD9*, could be eliminated in a double mutant. We have established that the origin-proximal portion of the peak has different genetic dependencies, but have not probed the nature of those dependencies further at this point.

- In figure 4 the author report the binding distribution of Dpb11 around early firing ARSs in replication stress conditions. It would be interesting to compare these results with Dpb11 binding in unstressed cells entering S phase.

Dpb11 binding at origins in cells entering S phase has been performed in the context of DNA replication studies, by ChIP-PCR, by the Araki lab (PMID: 10733584). Data in the Araki study indicated that Dpb11 associates with early origins in early S phase but is not detected on later replicating regions between the origins later in S, when replisome components can be detected on later replicating regions. We agree that it would be interesting to revisit Dpb11 binding in unperturbed S, but would prefer to leave that analysis to future studies that address how Dpb11 transitions from binding Sld2/3 in initiation to other binding events described by the Diffley, Smolka, and Pfander labs, and in our manuscript.

2nd Editorial Decision 28 May 2015

Thank you for submitting your revision to The EMBO Journal. Hartmut Vodermaier is the primary editor on the manuscript, but as he is away at the moment I have stepped in to help move things along.

Your manuscript has now been re-reviewed by referees #1 and 2 and their comments are provided below. As you can see, the referees appreciate the introduced changes and support publication here. Referee #1 has a few minor suggestions regarding the text that I would like you to take into consideration. You can send me a revised version by email and we will upload it for you.

If you have any questions please don't hesitate to contact me

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REFEREE COMMENTS

Referee #1

The authors have significantly improved their manuscript. They have addressed all the issues raised by the three reviewers and provide new data that make their case stronger. For instance, the new experiments showing the presence of Slx4 foci in MMS-treated and untreated S-phase cells are now convincing. The new model shown fig.7 is also very helpful. I only have a minor concern regarding the use of "CNV" to refer to changes in DNA copy number during the cell cycle. CNV is a widely-used abbreviation to refer to structural variations in the human genome. It is obvious that CNV refers here to replication-dependent copy number changes, but since this paper is one of the first to use this metrics to follow DNA replication, it would be more appropriate to use a different name. Moreover, the authors should cite earlier studies using DNA copy number variation to monitor replication, such as Yabuki (2002) Genes Cells 7, 781 (cited here for a different purpose) and Koren (2014) Cell 159, 1015.

Referee #2

The authors have satisfactorily answered most of my concerns/suggestions as well as those of the other referees with the addition of new high quality data and by making appreciable efforts to strengthen their discussion.

2nd Revision - authors' response 29 May 2015

As suggested by reviewer 1 we now refer to the CNV profiles as ‘replication profiles’, and we make reference to the two indicated papers in the Methods section. Thanks for your help with the review process.

