THE EMBO JOURNAL

Manuscript EMBO-2014-90834

Dampening DNA Damage Checkpoint Signalling via Coordinated BRCT Domain Interactions

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Review timeline:

Submission date:	17 December 2014
Editorial Decision:	29 January 2015
Revision received:	05 March 2015
Pre-acceptance letter:	24 March 2015
Accepted:	27 March 2015

Editor: Hartmut Vodermaier

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 29 January 2015

Thank you again for submitting your manuscript to our journal. I apologize that it has taken considerably longer than usual to get back to you with a decision, but with the end of the year holiday season, things had gotten delayed both in the editorial office and on part of the reviewers. We have now finally received the below comments from three expert referees, and in light of their assessment would like to invite you to prepare a revised version of the manuscript. As you will see, the referees raise a number of significant concerns, which would need to be satisfactorily clarified before eventual publication. In particular, it will be important to address the well-taken issues detailed by referee 2, as well as referee 1's query regarding the role of phosphorylation sites (see their point 2, "But what happens to[...]important mechanistic question to address"). In addition, please also take care of the various more specific/minor items listed in all three reports.

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me should you have any feedback or questions regarding the referee reports or this decision. I look forward to your revision.

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Referee #1:

The experiments and results presented in this manuscript are clearly of a good/high standard. However, much of the speculation and some of the conclusions drawn by the authors are of concern and require either some additional explanation or further experimental work.

It is also not totally clear how much of an advance (i.e. novelty) this manuscript makes over the work previously published by the same senior author (Ohouo et al, Nature 2013).

Major Points

(1) The authors propose that Rad9 displacement from the site of a lesion is used as a regulatory mechanism, in order to "down-regulate" DDC signalling, and that this is mediated through Rtt107-Slx4 interacting with pH2A and Dbp11.

There is clearly COMPETITION for binding to pH2A by both Rad9 and Rtt107; BRCT1/2 of Dpb11 can bind either to Rad9 or to Slx4; and Dpb11 is anchored to the 9-1-1 complex by BRCT3/4 - i.e. what the authors have demonstrated is that there is antagonism for access to the vicinity of a DNA lesion between the 'checkpoint signalling' and 'damage repair' complexes. But whether this actually constitutes a bona fide MECHANISM for 'checkpoint dampening' is still far from clear, and a matter for ongoing debate; especially as the function of the recruited Slx4-Rtt107 complex sits simply and firmly in DNA damage repair, rather than turning off Rad53 activity per se.

It is worth noting that over-expression experiments always have the intrinsic problem of perturbing / skewing an (often complex) equilibrium - and may therefore not truly reflect what would normally happen in the cell.

(2) The complex interactions made by the Dpb11 protein are, as the authors state, regulated by phosphorylation (and de-phosphorylation?) at different points of the cell-cycle and in direct response to the presence of DNA damage [in fact, the authors have already identified phosphorylation of Ser486 as important for the Dpb11/Slx4 interaction; presumably by binding to Dpb11-BRCT1/2?].

Furthermore, on page 12 of the discussion, and in Figure (6A) the authors indicate that the Rad9-Dpb11 interaction occurs early in S-phase (after alpha-factor release of synchronised cells into MMS-containing media), whilst the Slx4-Dpb11 interaction is only observable 25 minutes after release - with a concomitant drop in the amount of the Rad9-Dbp11 complex.

But what happens to the different phosphorylation sites driving these interactions? Are they still there? Have they actually been removed? This is an important mechanistic question to address.

The apparent antagonism between the Ser462/Thr474 phosphorylation sites in Rad9 and Ser486 of Slx4 warrants additional experimental work that should be included in a revised manuscript - especially as on page 16 of the discussion, the authors also state 'importantly, following checkpoint activation, we propose that Slx4-Rtt107 become more efficiently recruited and able to complete out Rad9 due to a presumably stronger interaction with Dpb11' - but without directly determining if this is actually the case.

Minor Points

(1) Page 4, 'recruitment of the yeast Rad9 adaptor has been shown to be mediated by three distinct mechanisms'; Page 4, 'notably, these three modes of adaptor recruitment are spatiotemporally distinct'

The H3K79 and pH2A marks are known to work as part of the same pathway; i.e. efficient recruitment of Rad9 and activation of checkpoint is dependant on BOTH marks - and therefore must exist at the same time and in the same place.

(2) Results, Page 5:
'BRCT domain recognise phosphorylated motifs in target proteins'.

Most BRCT domains do, in fact, recognised phosphorylated motifs, however, a number of them mediate non-phospho-mediated protein-protein interactions

Referee #2:

In the article "Dampening Checkpoint Signaling Via Coordinated BRCT-domain Interactions" the authors analyse molecular details of the mechanism that turns off activation of the DNA damage checkpoint at the transduction (Rad9) level. While the mechanism has been previously described (Ohouo 2013, Nature), this paper defines the precise binding events between BRCT domains and phosphorylated sites that are functionally required in this pathway. Moreover, by fusing domains of different proteins, the authors establish that the formation of these complexes is the only function carried out by these proteins in the DAMP pathway.

The quality of the manuscript is very good. In my view, this work is suited for publication in EMBO Journal after the authors have successfully addressed at least the majority of the following points:

1) Figure 2/ Figure 3: The authors show that a fusion between Dpb11 and the C-terminal domain of Slx4(S486A/7MUT) can suppress the MMS sensitivity of slx4 cells, and this depends on the functionality of the BRCT3/4 of Dpb11, which binds to Ddc1-T602. The authors should test if mutation of T602 can prevent the suppression of slx4 MMS sensitivity by the fusion protein, to conclusively show that this BRCT domain is binding to Ddc1 for the DAMP function and not to other phosphorylated substrates such as Sld2.

2) The authors show the suppression of the MMS sensitivity of slx4 cells by the Dpb11-Slx4 chimera and by the MBD, demonstrating that in MMS the sensitivity of those cells is only caused by lack of prompt checkpoint inactivation. Is this true for other drugs like phleomicin/zeocin or camptothecin or UV-induced damage? Do any of these drugs require additional functions of Slx4 that cannot be rescued by the syntetic constructs? The inclusion of such data would add to the work's impact.

3) It is not clear to me why slx4 cells die in the presence of MMS? Is it the checkpoint that is not timely switched off and thus impairs growth by arresting cell cycle progression? Or is it the fact that Mus81 is not properly activated as a consequence of the lack of checkpoint inactivation? Would overexpression of Mus81, or expression of a version of Mms4 that cannot be inhibited by the checkpoint, suppress the MMS sensitivity of an slx4 mutant?

4) Figure 3C: to conclusively demonstrate that overexpression of MBD rescues the delay in checkpoint inactivation, the authors should show kinetics of Rad53 phospho/dephosphorylation (as it is, it could be that the MBD simply impairs Rad53 activation).
5) Figure 6D: the authors should show a loading control for the immunoprecipitation (MBD-HA)

Minor comments:
Two typos in page 4: "phopho-H2Ais induced by DNA damage" and "by interacting with phopho-H2A and Dpb11"

Referee #3:

This is a remarkable study by Ohouo and colleagues. They present a thorough molecular analysis of the checkpoint dampening mechanism (DAMP) that they unraveled a couple of years ago. The experiments are well designed and the data are presented in a clear, meaningful and compelling way that support from biochemical, cellular and genetic standpoints their initial model.
This well crafted and matured study will undoubtedly be of interest to the DNA Damage Response field.

I have a few minor points listed below

Minor points:

- How would a BRCT1/2-BRCT5/6 MBD module behave? Could that not have been used at least as a negative control to answer some debate between the Smolka and Pfander labs around the question of how specific is the interaction between the BRCT1/2 and 3/4 of Dpb11 and SLX4 and Ddc1, respectively.
Along those lines, can the BRCT1/2 of Dpb11 bind Mms4?

- Remind what is the PATH tag so that the reader does not have to go back to the Ohouo et al. 2012 paper

- My understanding is that experiments described in Fig 1E were done in MMS. If so, this needs to be mentioned at least in the Figure legend

- In figure 4 the mmc1-delta mutant seems much less sensitive to HU than expected (see for example Ohouo et al 2012)

- Page 11 it is mentioned that a direct interaction between Dpb11 and Mms4 was reported by Gritenaite et al. 2014. Is that true? I believe that although this is likely there is no evidence that definitely proves a direct interaction.

- regarding Fig 6B, it is mentioned in the text 60 minutes after release of alpha factor arrested cells in MMS but in the figure legend it says cells treated with MMS for 2 hours.

1st Revision - authors' response 05 March 2015

We thank all the reviewers for the positive comments and the constructive suggestions. We have very carefully addressed the major and minor points as detailed below.

SPECIFIC RESPONSE TO REVIEWER #1:

MAJOR POINT 1: *"The authors propose that Rad9 displacement from the site of a lesion is used as a regulatory mechanism, in order to "down-regulate" DDC signalling, and that this is mediated through Rtt107-Slx4 interacting with pH2A and Dbp11.*

There is clearly COMPETITION for binding to pH2A by both Rad9 and Rtt107; BRCT1/2 of Dpb11 can bind either to Rad9 or to Slx4; and Dpb11 is anchored to the 9-1-1 complex by BRCT3/4 - i.e. what the authors have demonstrated is that there is antagonism for access to the vicinity of a DNA lesion between the 'checkpoint signalling' and 'damage repair' complexes. But whether this actually constitutes a bona fide MECHANISM for 'checkpoint dampening' is still far from clear, and a matter for ongoing debate; especially as the function of the recruited Slx4-Rtt107 complex sits simply and firmly in DNA damage repair, rather than turning off Rad53 activity per se.

It is worth noting that over-expression experiments always have the intrinsic problem of perturbing / skewing an (often complex) equilibrium - and may therefore not truly reflect what would normally happen in the cell."

We agree with the reviewer that overexpression can lead to artifacts and, in fact, this is the main reason why we chose our MBD chimera to be driven by the weak Dpb11 promoter. This is now explained in the results section by the following sentence: *"Importantly, MBD expression was driven by the relatively weak Dpb11 promoter [(Dpb11 protein is expressed at around 400 copies per cell; (Mantiero et al, 2011)] …."*

Furthermore, as shown in Figure 4, we also tested changing the MBD promoter to stronger promoters, which did result in MMS sensitivity, differently than the MBD driven by *DPB11*

promoter. This difference between low and high level of MBD expression is carefully explained in the results section.

MAJOR POINT 2: *"The complex interactions made by the Dpb11 protein are, as the authors state, regulated by phosphorylation (and de-phosphorylation?) at different points of the cell-cycle and in direct response to the presence of DNA damage [in fact, the authors have already identified phosphorylation of Ser486 as important for the Dpb11/Slx4 interaction; presumably by binding to Dpb11-BRCT1/2?].*

Furthermore, on page 12 of the discussion, and in Figure (6A) the authors indicate that the Rad9-Dpb11 interaction occurs early in S-phase (after alpha-factor release of synchronised cells into MMS-containing media), whilst the Slx4-Dpb11 interaction is only observable 25 minutes after release - with a concomitant drop in the amount of the Rad9-Dbp11 complex.

But what happens to the different phosphorylation sites driving these interactions? Are they still there? Have they actually been removed? This is an important mechanistic question to address.

The apparent antagonism between the Ser462/Thr474 phosphorylation sites in Rad9 and Ser486 of Slx4 warrants additional experimental work that should be included in a revised manuscript - especially as on page 16 of the discussion, the authors also state 'importantly, following checkpoint activation, we propose that Slx4-Rtt107 become more efficiently recruited and able to complete out Rad9 due to a presumably stronger interaction with Dpb11' - but without directly determining if this is actually the case.

These points are now carefully discussed in our model for how DAMP functions as a phosphatase-independent mechanism for counteracting Rad53 activation. We propose that a major advantage of the DAMP mechanism is the ability of antagonizing Rad53 activation in a localized manner and without the need of phosphatases (so no dephosphorylation is required). We have now included a highly detailed model for how the transitions in Slx4 and Rad9 interactions with Dpb11 are occurring (please see Fig E7). Also, we have included an additional time-point (45 minutes) in the temporal analysis of Dpb11-Rad9 and Dpb11-Slx4 interaction (shown in Fig. 6B) to further support the idea that Rad9 is not significantly dephosphorylated (gel shift of Rad9 still persists) although interaction with Dpb11 is strongly reduced.

Concerning strength of interactions, the model is also based on the finding that at least 10 times more Dpb11 is recovered from Slx4 IPs compared to Rad9 IPs (see Fig 6C). Importantly, we show that the total abundance of Rad9 in cell extracts is far higher than the abundance of Slx4 (Fig E1A), re-enforcing the idea that Slx4-Dpb11 interaction is more stable/stronger than the Rad9-Dpb11 interaction. We agree with the reviewer that direct analysis of dissociation constants of the different interactions will be useful and we are currently working on that. But given the fact that the mode of interactions are quite complex, requiring multiple phosphorylation sites in Slx4, we need first to tease apart all the sites and generate a panel of synthetic phosphopeptides for measuring interactions. We feel these analyses (which may take years to complete) are not critical to the proposition of the above model.

Minor Points

(1) Page 4, 'recruitment of the yeast Rad9 adaptor has been shown to be mediated by three distinct mechanisms'; Page 4, 'notably, these three modes of adaptor recruitment are spatiotemporally distinct'

The H3K79 and pH2A marks are known to work as part of the same pathway; i.e. efficient recruitment of Rad9 and activation of checkpoint is dependant on BOTH marks - and therefore must exist at the same time and in the same place.

We have now included further discussion and a more detailed working model depicting Rad9 and slx4-Rtt107 recruitment. Please refer to new expanded Figure E7 showing the model for establishment of histone marks. It is well established that methylation of H3K79 by the Dot1 methyltransferase occurs constitutively on chromatin (Nguyen & Zhang, 2011) while phosphorylation of H2A by Mec1 and Tel1 kinases is mostly induced at sites of DNA damage (Shroff et al, 2004). We believe the new detailed model in Figure E7 will better clarify the dynamics of histones marks and Rad9 recruitment.

(2) Results, Page 5: 'BRCT domain recognise phosphorylated motifs in target proteins'.

Most BRCT domains do, in fact, recognised phosphorylated motifs, however, a number of them mediate non-phospho-mediated protein-protein interactions

We agree this sentence needs to be re-structured for clarification. We have modified the sentence to *"BRCT domains <u>often</u> recognise phosphorylated motifs in target proteins'.*

SPECIFIC RESPONSE TO REVIEWER #2:

"In the article "Dampening Checkpoint Signaling Via Coordinated BRCT-domain Interactions" the authors analyse molecular details of the mechanism that turns off activation of the DNA damage checkpoint at the transduction (Rad9) level. While the mechanism has been previously described (Ohouo 2013, Nature), this paper defines the precise binding events between BRCT domains and phosphorylated sites that are functionally required in this pathway. Moreover, by fusing domains of different proteins, the authors establish that the formation of these complexes is the only function carried out by these proteins in the DAMP pathway.

The quality of the manuscript is very good. In my view, this work is suited for publication in EMBO Journal after the authors have successfully addressed at least the majority of the following points."

We thank the reviewer for the positive and interesting comments and we have addressed the points raised.

MAJOR POINT 1: *"Figure 2/ Figure 3: The authors show that a fusion between Dpb11 and the C-terminal domain of Slx4(S486A/7MUT) can suppress the MMS sensitivity of slx4Δ cells, and this depends on the functionality of the BRCT3/4 of Dpb11, which binds to Ddc1-T602. The authors should test if mutation of T602 can prevent the suppression of slx4Δ MMS sensitivity by the fusion protein, to conclusively show that this BRCT domain is binding to Ddc1 for the DAMP function and not to other phosphorylated substrates such as Sld2."*

This is a nice idea, but mutation of T602 to alanine already rescues the MMS sensitivity of *slx4Δ* cells (see Figure R1 below and (Gritenaite et al, 2014)), which is consistent with our model, as Dpb11 should not be recruited to Ddc1 and therefore not hyperactivate Rad53 via Rad9 interaction. To address the reviewer's point and show that BRCT domain is binding to Ddc1 for the DAMP we have mutated T602 to alanine and shown that in this mutant Slx4 can't co-immunoprecipitate with Ddc1 (Fig 1E), as predicted from our model that Dpb11 bridges Slx4 to Ddc1 via BRCT domain interactions.

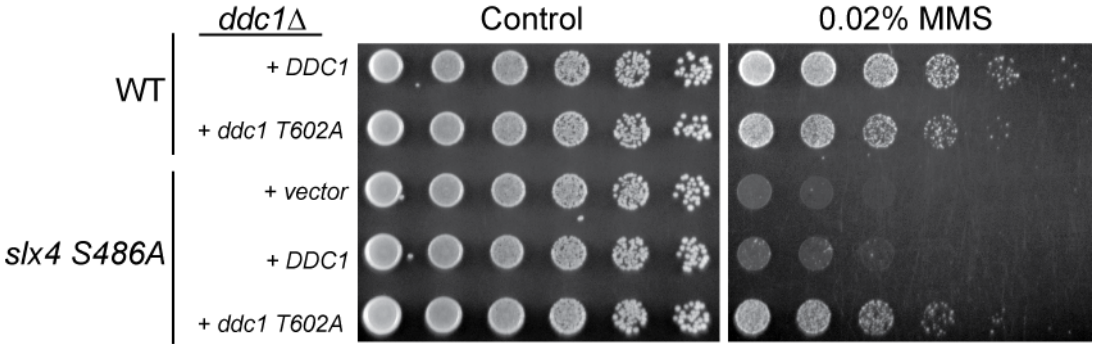

Figure R1. Ddc1 T602A rescues the MMS sensitivity of an *slx4-S486A* strain. Four-fold serial dilutions were spotted on plates and grown for 2-3 days in SD-URA plates at 30°C.

MAJOR POINT 2: "*The authors show the suppression of the MMS sensitivity of slx4Δ cells by the Dpb11-Slx4 chimera and by the MBD, demonstrating that in MMS the sensitivity of those cells is only caused by lack of prompt checkpoint inactivation. Is this true for other drugs like phleomicin/zeocin or camptothecin or UV-induced damage? Do any of these drugs require additional functions of Slx4 that cannot be rescued by the syntetic constructs? The inclusion of such data would add to the work's impact."*

Cells lacking Slx4 are not sensitive to drugs such as phleomicin/zeocin, CPT or HU (Gritenaite et al, 2014; Ohouo et al, 2013)), likely because these drugs fail to result in large amounts of ssDNA gaps behind a moving replication fork. However, it has been shown that an *slx4-S486A* strain has a mild sensitivity to 4-NQO (Gritenaite et al, 2014), a UV mimetic that, like MMS, generates bulky DNA lesions that stall the polymerase but can be bypassed by a moving replication fork leading to the generation of ssDNA gaps behind the fork. We performed a sensitivity assay in an *slx4*Δ strain and as expected, we observed a mild sensitivity to 4-NQO. Interestingly, this sensitivity could be rescued by expression of MBD (new Figure E3). Furthermore, to specifically address the very important question raised by the referee whether MBD construct rescues the checkpoint regulatory function but not other Slx4 functions, we examined if MBD specifically rescues the checkpoint regulatory function of Slx4 but not the established Slx4 function in DNA repair in conjunction with the structure-specific nuclease Slx1 (Fricke & Brill, 2003). For that, we examined if MBD is able to rescue the lethality caused by deletion of both SLX4 and SGS1, a helicase also known to display synthetic lethality with Slx1 (Mullen et al, 2001). Our prediction was that MBD would not rescue this synthetic lethality, for the following reasons: (1) while deletion of the Slx1 also results in synthetic lethality with *sgs1Δ*, Slx1 has no role in the DAMP mechanism (Ohouo et al, 2013); (2) Deletion of Rtt107, a protein that is required for DAMP (Fig 1A), does not result in lethality in *sgs1Δ* cells (Zappulla et al, 2005). Indeed, we found that expression of MBD does not rescue the lethality of cells lacking *SLX4* and *SGS1* (Fig 3G). Taken together, these results support our model that Slx4 has an important role in checkpoint down-regulation that is independent from its role in Slx1-mediated DNA repair.

MAJOR POINT 3: *"It is not clear to me why slx4Δ cells die in the presence of MMS? Is it the checkpoint that is not timely switched off and thus impairs growth by arresting cell cycle progression? Or is it the fact that Mus81 is not properly activated as a consequence of the lack of checkpoint inactivation? Would overexpression of Mus81, or expression of a version of Mms4 that cannot be inhibited by the checkpoint, suppress the MMS sensitivity of an slx4Δ mutant?"*

These are all excellent points. All of our generated data together with previous work by the Branzei lab (Szakal & Branzei, 2013) lead us to propose that *slx4Δ* cells die because of Rad53 hyperactivation that leads to inhibition of Mus81 action (likely by inactivation of Cdc5 activity needed for MMS4 phosphorylation and Mus81 activation). As mentioned by the reviewer, to further test this notion it would be ideal to generate an MMS4 mutant that bypasses the need of Cdc5 phosphorylation (and therefore should not be inhibited by checkpoint), but this mutant has been extremely hard to generate and, in principle, could still lead to hyperactive Mus81 in intra-S-phase, which would also lead to MMS sensitivity. Of note, overexpression of Mus81 unfortunately does not result in hyperactive Mus81. While work is currently in progress towards the generation of *mms4* mutants, we feel it would be more suitable for a separate study.

MAJOR POINT 4: *"Figure 3C: to conclusively demonstrate that overexpression of MBD rescues the delay in checkpoint inactivation, the authors should show kinetics of Rad53 phospho/dephosphorylation (as it is, it could be that the MBD simply impairs Rad53 activation)."*

It is important to mention that our model predicts that MBD may also impair Rad53 activation early in S-phase, and not only act during checkpoint inactivation. This is mainly because differently than the Slx4-Dpb11 interaction, which requires S-phase CDK activity to build up, MBD does not require such phosphorylation event and could, in principle, efficiently dock at lesions early in S-phase while S-phase CDK is still low (please see further explanation in the results section, under "Temporal dynamics of DAMP"). We have included the temporal analysis of Rad53 activation upon expression of MBD and overexpression with A-MBD (new Fig 6A). Interestingly, as predicted, we could observe impairment of Rad53 activation early in S-phase in cells expressing MBD (differently than the "physiological" Rtt107-Slx4-Dpb11 DAMP, which requires CDK phosphorylation for assembly and therefore dampens later in S-phase)..Furthermore, overexpression of MBD leads to a stronger impairment of Rad53 activation, explaining why it sensitizes even WT cells.

Taken together, these results reveal the distinct temporal dynamics between physiological DAMP (via phospho-dependent Slx4-Dpb11 interaction) and a "synthetic" DAMP (via MBD). Text has been modified in the results and discussion session to carefully explain this point.

MAJOR POINT 5: "*Figure 6D: the authors should show a loading control for the immunoprecipitation (MBD-HA)"*

We have added the loading control for the immunoprecipitation.

Minor comments: *"Two typos in page 4: "phopho-H2A is induced by DNA damage" and "by interacting with phopho-H2A and Dpb11"*

Corrected, thanks.

SPECIFIC RESPONSE TO REVIEWER #3:

"This is a remarkable study by Cussiol and colleagues. They present a thorough molecular analysis of the checkpoint dampening mechanism (DAMP) that they unraveled a couple of years ago. The experiments are well designed and the data are presented in a clear, meaningful and compelling way that support from biochemical, cellular and genetic standpoints their initial model.
This well crafted and matured study will undoubtedly be of interest to the DNA Damage Response field.

We thank the reviewer for the positive remarks and we have addressed the minor points raised.

I have a few minor points listed below
Minor points:

- How would a BRCT1/2-BRCT5/6 MBD module behave? Could that not have been used at least as a negative control to answer some debate between the Smolka and Pfander labs around the question of how specific is the interaction between the BRCT1/2 and 3/4 of Dpb11 and SLX4 and Ddc1, respectively.
Along those lines, can the BRCT1/2 of Dpb11 bind Mms4?

This is an important experiment that is shown in supplemental Fig E4. As expected by our model, this new MBD (herein named alt-MBD) failed to rescue the MMS sensitivity of an *slx4*Δ strain. Also, we added a new figure showing that while MBD is capable of interacting with Mms4, alt-MBD doesn't (Fig E6).

- Remind what is the PATH tag so that the reader does not have to go back to the Ohouo et al. 2012 paper

We have added detailed description of the PATH approach in the methods section under: "*Pull-down of recombinant BRCT domains using PATH tag*".

- My understanding is that experiments described in Fig 1E were done in MMS. If so, this needs to be mentioned at least in the Figure legend

Good point. We have added the information to the legend of Figure 1.

- In figure 4 the mmc1-delta mutant seems much less sensitive to HU than expected (see for example Ohouo et al 2012)

The difference in the level of HU sensitivity is likely because a different background strain was used here. As also reported by others, the HU sensitivity of mrc1Δ strains can vary substantially depending on the background [for other examples, please see here (Osborn & Elledge, 2003; Puddu et al, 2011)]. We were unable to use the *mrc1*Δ strains used in the Ohouo et al paper because the URA marker is not available so we could not transform our plasmids containing the MBD constructs. Nonetheless, we have reconfirmed the genotype of all the strains used in figure 4 by PCR and have repeated the experiments in other *mrc1*Δ strains in our collection, which gave the same results, so we are highly confident the results are correct.

- Page 11 it is mentioned that a direct interaction between Dpb11 and Mms4 was reported by Gritenaite et al. 2014. Is that true? I believe that although this is likely there is no evidence that definitely proves a direct interaction.

The reviewer is correct as it is not known until now if this interaction is direct or mediated by another protein. In our final model (Figure 7) we have now added an arrow pointing to the possibility for an indirect interaction between these two proteins. In addition, we added the following sentence to the discussion: "*Furthermore, it remains to be defined if BRCT 3/4 of Dpb11 is directly recognizing phosphorylated motifs in Mms4 or is actually binding Mms4 via another protein.*"

- regarding Fig 6B, it is mentioned in the text 60 minutes after release of alpha factor arrested cells in MMS but in the figure legend it says cells treated with MMS for 2 hours.

We apologize for the mistake as the correct information is that asynchronous cells were treated with MMS 0.04% for 60 minutes.

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Acceptance letter 27 March 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are satisfied with the revisions and raise no further objections towards publication in The EMBO Journal.

Referee #1

The manuscript has been greatly improved, and the authors' responses to referees' comments are in the main explanatory and appropriate.

I still have some general misgiving surrounding the notion of Slx4-Rtt107 recruitment (and its competition with Rad9) as a 'Dampening' mechanism per se; rather that downstream recruitment of this complex is in fact a tightly regulated phenomenon, that is intrinsic to the required downstream DNA damage repair processes.

However, given the positive responses from the other two referees, I accept that this manuscript is now suitable for publication in the EMBO Journal.

Referee #2

The authors have significantly strengthened their paper by addressing most of the issues raised by myself and the other referees regarding their first submission. In light of this, I am now able to recommend this article to be published in EMBO Journal

Referee #3

The authors have satisfactorily answered each of my concerns/questions. I have no further objection for this manuscript to be published in EMBO.

