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The cohesin complex is required for the DNA damage induced G2/M checkpoint in mammalian cells

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 20 March 2009

Thank you very much for submitting your manuscript for consideration to The EMBO Journal editorial office. From the enclosed reviewer comments you will see, that the three scientists agree on the novelty and merits of your findings. Based on their individual emphasis, all referees request certain modifications that will have to include additional experimental work. These are, however, solely scientifically justified and aimed at improving the quality and general impact of the paper. In particular ref#2 suggests valuable experiments that would also shed more light on the actual mechanism of cohesin mediated checkpoint activation (checking the impact of Chk2 phoshorylation; Scc1's role or the intra-S checkpoint for Smc1/3 phosphorylation?). Similarly, both ref#1 and #3 suggest to perform and/or repeat a few further knockdown analyses to significantly strengthen the overall conclusions that can be drawn from your work. Given this overall rather encouraging assessment I kindly invite you to address the concerns raised by our referees during one round of major amendments. I nevertheless do also have to remind you that it is EMBO_J policy to allow a single round of revisions only, which means that the final decision on acceptance or rejection depends on the content of the final version of your manuscript.

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

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Report on "The cohesin complex is required for the DNA damage induced G2/M checkpoint in mammalian cells" by Watrin and Peters

The cohesin complex or components of it have been implicated in DNA repair since the mid-nineties, and a role in DNA damage-induced checkpoint(s) was suggested thereafter. However, much of these particular functions of cohesin remains elusive. Watrin and Peters address the important question of specific checkpoint functions of cohesin, i.e. the G2/M and the intra-S checkpoints. They approach this problem in a sound, logical and systematic way and present novel insights. Among them, the independence of this checkpoint function from the sister chromatid cohesion activity, the impairment in cohesin-reduced cells of Chk2 activation and 53BP1 recruitment to damage-induced foci, and the need for cohesin in both checkpoints.

Before this important paper goes to press, I suggest the following modifications to perfect the paper:
1. In several instances (incl. in the abstract) the authors write "cells lacking cohesin". That is not entirely correct, since their Fig. 1 shows that upon RNAi treatment, some residual Scc1 and sororin are still present (and in Suppl. Fig 1A, some Smc3 is seen after Smc3- RNAi treatment).
2. Introduction, line 8 from bottom. The reference Sch‰r et al., 2004, who in S. cerevisiae showed control of repair through homologous recombination versus NHEJ by Smc1p, is missing.
3. Page 6, bottom: the authors state that Scc1 or Smc3 were depleted by RNAi and refer to Fig. 1B. However, Fig. 1B only shows Scc1 depletion, the Smc3 depletion is shown in Suppl. Fig. 1A.
4. Only in the Supplemental Figure 1 are data presented, which were derived from Smc3 RNAi treatments. These data differ in some respects from those obtained with Scc1 RNAi (e.g. in the extent of pulverized chromosomes upon irradiation). Thus, it would be informative to see more data on the effects of Smc3 RNAi, perhaps presented as additional supplemental figures. This is particularly important, since Smc3 is component also of non-cohesin complexes.
5. Figure 2 shows strong increase in gH2AX in Scc1 RNAi treated cells (see also Fig. 5B). Yet, there is no increase in gH2AX foci as reported on page 12. However, Suppl. Fig. 4 shows increased gH2AX staining in Scc1 RNAi-treated cells. The distinction in Suppl. Fig. 4C between discrete foci and "misshaped" foci is unclear, the legend superficial. Is there only an increase in more "diffuse" (= "misshaped"?) foci, or are there more foci and they are diffuse? Notably, gH2AX is not mentioned in the Discussion where Mdc1 is discussed (page 15, top). Thus, the situation regarding gH2AX is not clear from the paper as written. Also, the number of experiments/statistics is not adequately described.
6. Formally, it has not been published that Scc1 is excluded as a subunit of RC-1, as likely as that may be. Thus, the respective statement on page 9 may be modified like "Since Scc1 was not reported to be a subunit of RC-1...".
7. Figure 3A lacks a negative control (e.g. no ab) lane. I assume DNA damage was introduced by irradiation, and the legend should say so. "Xt" needs to be explained in the legend (many legends are "minimalistic"). The label "DSBs" is not entirely correct, since irradiation does not exclusively produce DSBs. It should say something like "irrad."
8. Since the difference between control and Scc1 RNAi samples in Figure 3C is small, and of limited statistical significance, details should be provided on the calculation: for example, were all six samples (2 x triplicates) averaged or were averages of each set of triplicates compared?
9. Is there a particular reason, why the Smc3S1083P lane is missing from the "etoposide blot" in Figure 4A?

Can the authors state that Smc1 and Smc3 are phosphorylated only as part of cohesin, or would they consider additional complexes within which these proteins can be phosphorylated in response to DNA damage at the respective residues? The authors may like to brefly discuss this.

It is unknown, which fraction of the cells' total cohesin serves in DNA repair, in checkpoint control, in sister chromatid cohesion, whether, for example, it is the tightly or the loosely chromatin associated cohesin fraction, whether there are transient protein associations distinguishing cohesin-like complexes (perhaps incl. the ATM-induced complex), or whether "the one cohesin" can do it all, if properly posttranslationally modified. These aspects may be more deeply discussed towards the end of the paper.

Throught the text, there are recurring typos where a comma would be needed in front of of sentence starting with "which".
Page 15, top: typo 53BPP1.

Referee #2 (Remarks to the Author):

Watrin and Peters paper:

In this paper, the authors investigate the function of cohesin in activating the G2/M damage induced checkpoint. They find that RNAi knockdown of the core cohesin complex members, Smc3 and Scc1 result in a failure to activate the G2/M checkpoint. Alternatively, Soronin knockdown results in a defect in DSB repair, but not in checkpoint activation. Furthermore, the authors show that Scc1 is required for phosphorylation of Smc1/Smc3 in the intra-S-checkpoint, suggesting that Smc1/3 phosphorylation occurs in the context of cohesin, rather than as a part of a distinct recombination complex RC-1. Interestingly, the authors propose the mechanism by which cohesin activates the checkpoint is independent of its role in cohesion. Finally, the authors propose a mechanism by which cohesin recruits checkpoint proteins (specifically 53B1) to the sites of DSBs, resulting in a failure to activate the checkpoints (possibly through Chk2 phosphorylation.)

Overall this paper is well written, and presents some interesting new observations. The paper could be strengthened, however, by providing further experiments to get at the mechanism for how cohesin activates the checkpoint. For example, the authors could see whether a phosphomimic of Chk2 T68 could rescue the checkpoint defect of cohesin RNAi cells. Furthermore, it is not clear what is known in the literature about T68 phosphorylation and its role in activation of the corresponding checkpoint.

The authors make the interesting observation that Smc1 and Smc3 phosphorylation is eliminated upon Scc1 knockdown. It would be interesting to know if the failure to activate the checkpoint in Scc1 knockdown cells is a direct consequence of a failure to phosphorylate this proteins. Again, this could be tested with phosphomimics. Alternatively, it is not clear to the reviewer whether the intra-S checkpoint is required for these phosphorylation events. If it were required, this would complicate the authors interpretation that the phosphorylation events must occur in the context of cohesin, because the lack of phosphorylation could be the result of indirect failure to activate the checkpoint, and the protein(s) required for the phosphorylation response.

To further substantiate the findings that 53B1 localization in perturbed in cohesin mutants, RNAi knockdown of another cohesin subunit (such as Smc3) should be looked at.

Finally, we have a few other minor comments which should be addressed:

1) Sororin should be introduced in the introduction
2) A citation is missing for cohesin recruitment to the sites of DSBs in the introduction
3) The authors should at least comment on why cohesin knockdown results in massive amounts of H2AX phosphorylation even in the absence of irradiation
4) It is not clear from the text whether 53BP1 is required for all Chk2 phosphorylation, or just T68. Since the authors only see a defect in T68, this is important to note.

Referee #3 (Remarks to the Author):

In the submitted manuscript Drs Watrin and Peters show in a convincing way that cohesin, but not full cohesion, is needed for the DNA damage induced G2/M checkpoint. In it shown that depletion of the cohesin subunits Scc1 or Smc3 cause a defective cell cycle arrest in response to two different damaging agents. In contrast, depletion of sororin which to generates a cohesion defect without affecting the chromosomal association of cohesin, leaves the checkpoint unperturbed. The already identified roles of Smc3 and Smc1 in S-phase checkpoint activation are also shown to be executed in the context of the cohesin complex. Screening of several DNA damage response factors determines that depletion of Scc1 not only inhibits phosphorylation of Smc1 and 3 in response to damage, but also reduces a specific activating phosphorylation of the signaling kinase Chk2. Cohesin depletion also interferes with the association of the damage response transducer 53BP1 to DNA repair foci. 53BP1 have been suggested to be involved in Chk2 activation (although this is not undisputed as pointed out by the authors). Based on this, Drs Watrin and Peters suggest that cohesin's role in checkpoint activation is to facilitate the recruitment of checkpoint proteins to the site of damage. A role of cohesin in the efficient recruitment of proteins to the site of damage is

further supported by an irregular shape of repair foci in Scc1 depleted cells. The investigation is made in a precise and thorough way and reveals new information on the function of cohesin in the DNA damage response. I consider that the manuscript definitively merits publication in EMBO journal. However, the following points need to be addressed.

As pointed out by the authors, figure 4 shows that ATM and Chk1 activation in response to damage is increased Scc1-depleted cells. The reason for this, and the possible effect of this increased activation, should be more elaborately discussed upon. In addition, ATM, Chk1 and -H2AX are phosphorylated even before damage induction in the Scc1 depleted cells (Fig 2 and 4). Why, and could this influence the results or their interpretation?

Since the experiments shown in figure 6 and S4 are critical for the conclusion drawn by the authors, the classification of foci-containing cells (Class I - III) could be more clearly defined in the text. Graphs with standard deviations would also facilitate the evaluation of data.

The conclusion that the role of cohesin in checkpoint activation is executed at the damaged chromosome could be tested further in an experiment using NIPBL/Scc2 depletion, which interferes with cohesin's chromosomal association, but not the stability of cohesin.

Minor points:

On page 3, fifth line from the bottom, it is stated that "cohesin establishes novel cohesive structures both at the DSB sites and throughout the genome". To my knowledge, even though cohesin is recruited to the site of damage, the formation of cohesion directly at a DSB has not yet been tested.

On page 5, "for" is missing between "required" and "the"

1st Revision - authors' response 18 June 2009

Reply to the comments of the Referees

We would like to thank all three Referees for their insightful and constructive suggestions which have clearly helped us to improve our manuscript.

Referee #1 (Remarks to the Author):

Report on "The cohesin complex is required for the DNA damage induced G2/M checkpoint in mammalian cells" by Watrin and Peters

The cohesin complex or components of it have been implicated in DNA repair since the mid-nineties, and a role in DNA damage-induced checkpoint(s) was suggested thereafter. However, much of these particular functions of cohesin remains elusive. Watrin and Peters address the important question of specific checkpoint functions of cohesin, i.e. the G2/M and the intra-S checkpoints. They approach this problem in a sound, logical and systematic way and present novel insights. Among them, the independence of this checkpoint function from the sister chromatid cohesion activity, the impairment in cohesin-reduced cells of Chk2 activation and 53BP1 recruitment to damage-induced foci, and the need for cohesin in both checkpoints.

Before this important paper goes to press, I suggest the following modifications to perfect the paper:
1. In several instances (incl. in the abstract) the authors write "cells lacking cohesin". That is not entirely correct, since their Fig. 1 shows that upon RNAi treatment, some residual Scc1 and sororin are still present (and in Suppl. Fig 1A, some Smc3 is seen after Smc3- RNAi treatment).

The phrase "cells lacking cohesin" has now been replaced by the phrase "after RNAi-mediated depletion of cohesin" to indicate that depletion was achieved by RNAi, which typically is incomplete.

2. Introduction, line 8 from bottom. The reference Schär et al., 2004, who in S. cerevisiae showed control of repair through homologous recombination versus NHEJ by Smc1p, is missing.

The missing reference Schär et al., 2004 has now been added to the Introduction.

3. Page 6, bottom: the authors state that Scc1 or Smc3 were depleted by RNAi and refer to Fig. 1B. However, Fig. 1B only shows Scc1 depletion, the Smc3 depletion is shown in Suppl. Fig. 1A.

The Supplemental Figure has now been cited at the appropriate position in the Results section.

4. Only in the Supplemental Figure 1 are data presented, which were derived from Smc3 RNAi treatments. These data differ in some respects from those obtained with Scc1 RNAi (e.g. in the extent of pulverized chromosomes upon irradiation). Thus, it would be informative to see more data on the effects of Smc3 RNAi, perhaps presented as additional supplemental figures. This is particularly important, since Smc3 is component also of non-cohesin complexes.

We have performed additional Smc3 RNAi experiments to address the Referee's concern and have included the resulting data in two new Supplemental Figures. Supplemental Figure 5 shows that depletion of Smc3 also reduces the levels of Chk2 phosphorylation on T68, and Supplemental Figure 7C shows that Smc3 depletion also decreases the recruitment of 53BP1 into IRIFs.

However, the Referee is correct that Smc3 depletion causes less strong phenotypes than depletion of Scc1. We suspect that this is due to less efficient depletion of Smc3 (approximately 75%, see semi-quantitative Western blot data in Supplemental Figure 5B) than of Scc1 (more than 90%, see Figure 4B). We have now explained this in the Results section on pages 11 and 14.

5. Figure 2 shows strong increase in gH2AX in Scc1 RNAi treated cells (see also Fig. 5B). Yet, there is no increase in gH2AX foci as reported on page 12. However, Suppl. Fig. 4 shows increased gH2AX staining in Scc1 RNAi-treated cells.

The Referee is correct that our description of the gH2AX results was somewhat misleading in that it sounded as if gH2AX staining was unaffected in cells depleted of cohesin. What we wanted to say was that gH2AX staining is not reduced in cells depleted of cohesin. We have corrected this statement, and have now also pointed out that gH2AX staining is in fact increased in cells depleted of cohesin (p12 and p16; see also below).

The distinction in Suppl. Fig. 4C between discrete foci and "misshaped" foci is unclear, the legend superficial. Is there only an increase in more "diffuse" (= "misshaped"?) foci, or are there more foci and they are diffuse? Notably, gH2AX is not mentioned in the Discussion where Mdc1 is discussed (page 15, top). Thus, the situation regarding gH2AX is not clear from the paper as written. Also, the number of experiments/statistics is not adequately described.

If one tries to count the number of IRIFs in Scc1 depleted cells it appears as if their number is not clearly increased, but the area occupied by each focus is. However, it is impossible to know if the larger IRIFs in Scc1 depleted cells are in reality not clusters of multiple foci which overlap due to their increased diameter. We have discussed this on p13 of the manuscript, although only briefly because we presently do not know if this phenomenon is of physiological relevance, and it does, therefore, not represent a central part of our study.

The IRIF structure is now described in more detail in the legend for this Figure (previously Supplemental Figure 4, now Supplemental Figure 6), and information on the number of experiments performed and displayed has also been added.

6. Formally, it has not been published that Scc1 is excluded as a subunit of RC-1, as likely as that may be. Thus, the respective statement on page 9 may be modified like "Since Scc1 was not reported to be a subunit of RC-1...".

This is a good point, and we have now stated on p9 that Scc1 has not been reported to be a subunit of RC-1.

7. Figure 3A lacks a negative control (e.g. no ab) lane. I assume DNA damage was introduced by irradiation, and the legend should say so. "Xt" needs to be explained in the legend (many legends are "minimalistic"). The label "DSBs" is not entirely correct, since irradiation does not exclusively produce DSBs. It should say something like "irrad."

We had not included a negative control for antibody specificity because we had extensively characterized the cohesin antibodies used for these experiments in previous publications (Sumara et al., 2002; Hauf et al., 2005). However, the Referee is right that negative controls are always important, and we have, therefore, repeated these experiments and included control IgGs as a negative control. These new results are shown in Supplemental Figure 3

The abbreviation "Xt" is now explained in the legend. As DNA damage was induced by etoposide we also replaced "DSBs" by "etoposide".

8. Since the difference between control and Scc1 RNAi samples in Figure 3C is small, and of limited statistical significance, details should be provided on the calculation: for example, were all six samples (2 x triplicates) averaged or were averages of each set of triplicates compared?

We have now described in more detail in the Figure legend how the data were obtained (two experiments were done, each in triplicate; the results of all six measurements were averaged).

9. Is there a particular reason, why the Smc3S1083P lane is missing from the "etoposide blot" in Figure 4A?

There was no particular reason. The samples from this experiment were simply not analysed for Smc3S1083ph. However, other experiments showed that Smc3S1083ph is strongly reduced after Scc1 or Smc3 RNAi (see Supplemental Figure 5A).

Can the authors state that Smc1 and Smc3 are phosphorylated only as part of cohesin, or would they consider additional complexes within which these proteins can be phosphorylated in response to DNA damage at the respective residues? The authors may like to brefly discuss this.

The Referee is correct that we can formally not exclude that other Smc1/Smc3 containing complexes exist. However, mass spectrometric analysis of Smc3 immunoprecipitates has not provided any evidence for the existence of such complexes in HeLa cells. If they exist, they would, therefore, either have to be of low abundance or to be too labile to persist during the isolation procedure. We have now briefly discussed this issue on pages 9 and 10.

It is unknown, which fraction of the cells' total cohesin serves in DNA repair, in checkpoint control, in sister chromatid cohesion, whether, for example, it is the tightly or the loosely chromatin associated cohesin fraction, whether there are transient protein associations distinguishing cohesin-like complexes (perhaps incl. the ATM-induced complex), or whether "the one cohesin" can do it all, if properly posttranslationally modified. These aspects may be more deeply discussed towards the end of the paper.

These are interesting questions, but the truth is that there is little we can say about the possible answers, even on a speculative level. We have, therefore, not raised these questions explicitly in the manuscript, with the exception that we have pointed out on page 17 that "non-cohesive" binding of cohesin to DNA (which is known to be dynamic, Gerlich et al., 2006; Schmitz et al., 2007) is apparently sufficient for the function of cohesin in DNA damage checkpoints.

Through the text, there are recurring typos where a comma would be needed in front of sentence starting with "which".

We have carefully checked the revised manuscript, corrected typographical errors and added commas, where appropriate.

Page 15, top: typo 53BPP1.

This error has been corrected.

Referee #2 (Remarks to the Author):

Watrin and Peters paper:

In this paper, the authors investigate the function of cohesin in activating the G2/M damage induced checkpoint. They find that RNAi knockdown of the core cohesin complex members, Smc3 and Scc1 result in a failure to activate the G2/M checkpoint. Alternatively, Soronin knockdown results in a defect in DSB repair, but not in checkpoint activation. Furthermore, the authors show that Scc1 is required for phosphorylation of Smc1/Smc3 in the intra-S-checkpoint, suggesting that Smc1/3 phosphorylation occurs in the context of cohesin, rather than as a part of a distinct recombination complex RC-1. Interestingly, the authors propose the mechanism by which cohesin activates the checkpoint is independent of its role in cohesion. Finally, the authors propose a mechanism by which cohesin recruits checkpoint proteins (specifically 53B1) to the sites of DSBs, resulting in a failure to activate the checkpoints (possibly through Chk2 phosphorylation.)

Overall this paper is well written, and presents some interesting new observations. The paper could be strengthened, however, by providing further experiments to get at the mechanism for how cohesin activates the checkpoint.

For example, the authors could see whether a phosphomimic of Chk2 T68 could rescue the checkpoint defect of cohesin RNAi cells. Furthermore, it is not clear what is known in the literature about T68 phosphorylation and its role in activation of the corresponding checkpoint.

We did consider such rescue experiments very seriously already early in the project. However, studies on Chk2 "knockout" mice came to somewhat conflicting conclusions about the importance of Chk2 in the G2/M checkpoint. Hirao et al. (Science 2000) reported that Chk2 -/- ES cells maintained irradiation induced arrest after 12 hours, but in contrast to Chk2+/+ cells not after 18 hours, whereas Takai et al. (EMBO J. 2002) could not detect defects in the G2/M checkpoint in Chk2-/- ES cells and embryonic fibroblasts. To address if Chk2 is required for this checkpoint in HeLa cells we depleted the protein by RNAi. We could not detect defects in the G2/M checkpoint. Although we cannot exclude that this result might have been caused by incomplete depletion, it is consistent with the mouse studies which observed that the G2/M checkpoint is largely intact in the absence of Chk2. For these reasons we consider it unlikely that lack of Chk2 activation is the main cause of the checkpoint defect that is observed in cohesin depleted cells (the defects in 53BP1 localization might be more important). Please note that we already stated this very clearly in the original version of the manuscript (see p16 of the revised version). For these reasons we decided not to perform rescue experiments with Chk2T68-phospho-mimetic mutants, apart from the fact that such experiments are difficult to control. However, this is not to say that the reduction in Chk2T68 phosphorylation may not be relevant. It is likely not the only cause of the checkpoint defect in cohesin depleted cells, as we explained in the manuscript, but it is a clear indication that checkpoint signalling is defective in these cells.

The authors make the interesting observation that Smc1 and Smc3 phosphorylation is eliminated upon Scc1 knockdown. It would be interesting to know if the failure to activate the checkpoint in Scc1 knockdown cells is a direct consequence of a failure to phosphorylate this proteins. Again, this could be tested with phosphomimics.

This is an interesting question, but for practical reasons we believe that it is difficult to test if expression of phospho-mimetic SMC proteins can "rescue" the effect of Scc1 depletion. The problem is that Smc1 and Smc3 can only interact with chromatin in the presence of Scc1 (see, for example, Watrin et al., Curr. Biol. 2006). Since cohesin is recruited to DSB sites (Bekker-Jensen et al., J. Cell Biol. 2006; Potts et al., EMBO J. 2006) and is needed to efficiently recruit 53BP1 to these sites (this manuscript) it is likely that phosphorylated Smc1 and Smc3 have to interact with DNA to function in checkpoint signalling. Since this is not possible in Scc1 depleted cells the prediction is that expression of phospho-mimicking Smc1/Smc3 could not rescue the Scc1 depletion phenotype, and we did, therefore, not perform these experiments.

Alternatively, it is not clear to the reviewer whether the intra-S checkpoint is required for these phosphorylation events. If it were required, this would complicate the authors interpretation that the phosphorylation events must occur in the context of cohesin, because the lack of phosphorylation could be the result of indirect failure to activate the checkpoint, and the protein(s) required for the phosphorylation response.

The Referee is correct that our finding that Scc1 depletion abolishes Smc1 and Smc3 phosphorylation is consistent with two different scenarios. It is possible that lack of Scc1 inactivates the intra-S phase checkpoint and that, therefore, Smc1 and Smc3 can not be phosphorylated. Alternatively, it is possible that Smc1 and Smc3 have to be recruited to DSBs to be phosphorylated, and since Scc1 is needed for DNA association of Smc1/Smc3 (see above) Scc1 depletion could prevent Smc1/Smc3 phosphorylation by preventing their recruitment to DSBs.

These two possibilities are not mutually exclusive, but we favor the second possibility for several reasons. First, ATM has been reported to be required for Smc1 phosphorylation (Kim et al., Genes Dev. 2002; Yazdi et al., Genes Dev. 2002), but ATM is still phosphorylated on S1981 in Scc1 depleted cells (this manuscript), indicating that Scc1 depletion does not prevent ATM activation. Likewise, Chk1 and H2AX are phosphorylated in Scc1 depleted cells, and MRN, RPA and Mdc1 are recruited to IRIFs. At least as defined by these criteria, the intra-S phase checkpoint can, therefore, still be activated in Scc1 depleted cells. Second, since phosphorylated forms of Smc1/Smc3 accumulate at DSBs (Bekker-Jensen et al., J. Cell Biol., 2006) and since the binding of Smc1/Smc3 to DNA depends on Scc1 (see above) we consider it more plausible to think that Scc1 depletion abolishes Smc1/Smc3 phosphorylation by preventing their recruitment to DSBs.

To further substantiate the findings that 53B1 localization in perturbed in cohesin mutants, RNAi knockdown of another cohesin subunit (such as Smc3) should be looked at.

This was an excellent suggestion. We, therefore, performed additional Smc3 RNAi experiments and found that Smc3 depletion also decreases the recruitment of 53BP1 into IRIFs, although to a somewhat lesser extent than Scc1 depletion. We suspect that this is due to less efficient depletion of Smc3 (approximately 75%, see semi-quantitative Western blot data in Supplemental Figure 5B) than of Scc1 (more than 90%, see Figure 4B). The new data on 53BP1 localization in Smc3 depleted cells are shown in Supplemental Figure 7C.

Finally, we have a few other minor comments which should be addressed:

1) Sororin should be introduced in the introduction

We have now introduced sororin more explicitly in the Introduction on p4.

2) A citation is missing for cohesin recruitment to the sites of DSBs in the introduction

The missing citation (Bekker-Jensen et al, 2006) has been added.

3) The authors should at least comment on why cohesin knockdown results in massive amounts of H2AX phosphorylation even in the absence of irradiation

We have now described the increase in phosphorylation of H2AX, as well as that of ATM and Chk1, in cohesin depleted cells in the Results section on p12, and have discussed the implications of this finding in the Discussion on pages 16 and 17.

4) It is not clear from the text whether 53BP1 is required for all Chk2 phosphorylation, or just T68. Since the authors only see a defect in T68, this is important to note.

In the literature, only Chk2 phosphorylation at T68 has been shown to depend on 53BP1. We have now described this explicitly in the text. To our knowledge, no data have been reported about roles of 53BP1 in phosphorylation of Chk2 at other sites.

Referee #3 (Remarks to the Author):

In the submitted manuscript Drs Watrin and Peters show in a convincing way that cohesin, but not full cohesion, is needed for the DNA damage induced G2/M checkpoint. In it shown that depletion of the cohesin subunits Scc1 or Smc3 cause a defective cell cycle arrest in response to two different damaging agents. In contrast, depletion of sororin which to generates a cohesion defect without affecting the chromosomal association of cohesin, leaves the checkpoint unperturbed. The already identified roles of Smc3 and Smc1 in S-phase checkpoint activation are also shown to be executed in the context of the cohesin complex. Screening of several DNA damage response factors determines that depletion of Scc1 not only inhibits phosphorylation of Smc1 and 3 in response to damage, but also reduces a specific activating phosphorylation of the signalling kinase Chk2. Cohesin depletion also interferes with the association of the damage response transducer 53BP1 to DNA repair foci. 53BP1 have been
suggested to be involved in Chk2 activation (although this is not undisputed as pointed out by the authors). Based on this, Drs Watrin and Peters suggest that cohesin's role in checkpoint activation is to facilitate the recruitment of checkpoint proteins to the site of damage. A role of cohesin in the efficient recruitment of proteins to the site of damage is further supported by an irregular shape of repair foci in Scc1 depleted cells. The investigation is made in a precise and thorough way and reveals new information on the function of cohesin in the DNA damage response. I consider that the manuscript definitively merits publication in EMBO journal. However, the following points need to be addressed.

As pointed out by the authors, figure 4 shows that ATM and Chk1 activation in response to damage is increased Scc1-depleted cells. The reason for this, and the possible effect of this increased activation, should be more elaborately discussed upon. In addition, ATM, Chk1 and γ-H2AX are phosphorylated even before damage induction in the Scc1 depleted cells (Fig 2 and 4). Why, and could this influence the results or their interpretation?

We have now described more explicitly on p12 of the Results section that phopshorylation of ATM, Chk1 and H2AX is increased in cohesin depleted cells, not only after induction of DNA damage but even before that. We have discussed the possible implications of these findings in the on pages 16 and 17 in the Discussion section. In brief, we suspect that this phenomenon is caused, at least in part, by the accumulation of DNA DSBs that occur spontaneously in these cells, consistent with cohesin's known role in DNA damage repair. Similarly, it has been observed before that depletion of Chk1 causes an increase in phosphorylation of H2AX and other DNA damage proteins (Syljuåsen et al., Mol. Biol. Cell, 2005). In addition, it is conceivable that a strong reduction in cohesin levels on chromatin may not only prevent the efficient repair of DSBs but may also make DNA more susceptible to the formation of damage. However, clearly more work will be needed to understand the molecular cause of this phenomenon.

"could this influence the results or their interpretation?":

This is a good question. Given the observed increase in ATM, Chk1 and H2AX phosphorylation we would expect that checkpoint signalling is activated more strongly in cohesin depleted cells than in cohesin proficient cells. Since cohesin depletion, nevertheless, causes substantial defects in DNA damage checkpoints the implication is that cohesin must have a particularly important role in these checkpoint pathways. We have now also discussed these considerations on pages 16 and 17 of our revised manuscript.

Since the experiments shown in figure 6 and S4 are critical for the conclusion drawn by the authors, the classification of foci-containing cells (Class I - III) could be more clearly defined in the text. Graphs with standard deviations would also facilitate the evaluation of data.

The classification of 53BP1 foci is now more precisely described in both the legend of Figure 6 and the Results section. In addition, we have performed additional experiments to test if 53BP1 localization is not only altered in Scc1 depleted cells but also in cells depleted of Smc3. These new data are shown in Supplemental Figure 7C, and graphs with standard deviations have been added to the data shown in Figure 6 and in Supplemental Figure 7C. For a further discussion of these data please see also response to comment No. 4 of Referee #1.

The conclusion that the role of cohesin in checkpoint activation is executed at the damaged chromosome could be tested further in an experiment using NIPBL/Scc2 depletion, which interferes with cohesin's chromosomal association, but not the stability of cohesin.

Also this is an excellent idea, and we have indeed tried (already before submission of the original version) to address this question, but so far without being able to reach a clear conclusion. Depletion of NIPBL/Scc2 or Scc4 reduces Smc1 phosphorylation and checkpoint function, but not as dramatically as Scc1 depletion. It is well possible that incomplete Scc2/Scc4 depletion and residual cohesin loading onto chromatin is responsible for these results, but much more work will be needed to clarify this and to exclude other possible explanations. An additional complication is that we are presently not able to deplete NIPBL/Scc2 in a way that would allow us to separate loading of cohesin onto chromatin in telophase from de novo loading of cohesin onto chromatin following DNA damage, which would be interesting to do. In conclusion, although this is a very interesting question it is unfortunately beyond the scope of this manuscript to answer it.

Minor points:

On page 3, fifth line from the bottom, it is stated that "cohesin establishes novel cohesive structures both at the DSB sites and throughout the genome". To my knowledge, even though cohesin is recruited to the site of damage, the formation of cohesion directly at a DSB has not yet been tested.

The text has been amended accordingly.

On page 5, "for" is missing between "required" and "the"

The typo has been corrected.

