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hPrimpol1/CCDC111 is a human DNA primase-polymerase required for the maintenance of genome integrity

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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.)

Editor: Esther Schnapp

1st Editorial Decision 12 August 2013

Thank you for the submission of your research manuscript to our journal. We have now received the enclosed reports on it.

As you will see, all referees agree that the findings are potentially interesting and important. However, they also raise concerns and have several suggestions for how the study could be improved. Both referees 1 and 3 indicate that pCHK2 levels should be investigated after hPrimpol1 depletion, and referee 3 adds that the presence of pATM and 53BP1 should be analyzed as well, in order to support the hypothesis that hPrimpol1 depletion generates DSB. Referee 2 further points out that it should be examined in which phase of the cell cycle the DNA damage foci occur after hPrimpol1 knockdown. Other concerns regard hPrimpol1 antibody characterization, looking at endogenous hPrimpol1, data quantification and discussion.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of

revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any further questions or comments regarding the revision.

Referee #1:

Summary
1. This manuscript presents a structure-function study of a novel human protein hPrimpol1 of the archaeal-eukaryotic primase family.
2. The authors have mapped a direct physical interaction of recombinant hPrimpol1 with human RPA70 subunit in vitro, and show using appropriate controls that the two proteins localize in subnuclear DNA damage foci in human (HeLa) cells exposed to HU or ionizing radiation. They further demonstrate that hPrimpol1 has intrinsic primase and DNA polymerase activities that can be inactivated by single residue substitutions. Lastly, they show using shRNA-depleted HeLa and knockdown cells reconstituted with silencing-resistant hPrimpol1 and mutants that both the primase activity and the interaction with RPA contribute to the ability of cells to recover from replication stress in several different assays.
3. Taken together the data suggest a potentially very significant role for this protein in counteracting replication stress in cultured cells.
4. These findings are of general interest to the molecular biology community and more broadly in biological evolution. They are of special interest to genome stability and cancer researchers.

Questions/suggestions that should be addressed:
5. Fig. 5C (and 6C). The legend mentions no treatment of cells with HU or other damage-inducing agent, suggesting that depletion of hPrimpol1 was sufficient by itself to induce robust gH2AX foci in the indicated % of the cells - correct?

6. Fig. 5J. A simple depletion of hPrimpol1 in lanes 2-3 was not sufficient to induce phospho-Chk1 signaling in the absence of HU. Did the authors also test phospho-Chk2? What checkpoint kinase responds in the absence of induced damage to generate gH2AX in Figure 5C and 6C, but not in 5J?

7. Fig. 7E DNA fiber images, except for the vector image, do not seem to correlate well with the bar graphs shown in 7F. The authors should provide the detailed data for each sample (number of tracts of each length measured) in a supplement to justify the bar graphs shown here. The methods on p. 21-2 provide no information on the quantification.

8. Discussion. The authors have so far not noted the existence of conserved re-priming pathways among prokaryotes and the question of whether such pathways exist among eukaryotes. The impact of their findings might be heightened by a brief mention of what is known about re-priming.

Minor clarifications
Fig. 1 legend, panel E. What do the authors mean by Morc3?
Fig. 1, panel I legend. Do the authors mean to say that the cells were exposed to 2 mM HU for a 16 h time period and to 3 Gy IR for a 3 h time period?

Referee #2:

This article presents the identification of CCDC111 (renamed hPrimpol1 by the authors) as a replication stress response protein that localizes to stalled forks via an interaction with RPA. The authors report that purified Primpol1 has primase and DNA polymerase activity. Knockdown with

shRNA causes an increase in H2AX phosphorylation and a defect in recovery from replication stress. Overall the data support the authors conclusions and the results are significant and interesting. I offer only minor comments for improvement.

1. The authors state that RPA binding to the C-terminal region of Primpol1 does not influence its enzymatic activity. Since those biochemical assays were done in the absence of RPA, the authors need to qualify their conclusions.

2. Presumably the increase in H2AX when Primpol is knocked down happens due to problems in S-phase. It would be useful to document this experimentally.

3. The interaction of many proteins with RPA1 have been documented extensively and a consensus binding motif described (see Xu et al., MCB 2008 for example). It would be useful for the authors to examine the C-terminal domain of Primpol for this binding motif.

4. Please remove the word "specifically" from the sentence on page 11 describing the shRNAs. It is very unlikely that any shRNAs are completely "specific" to one gene. The documentation that the effects are not off-target is sufficient but there is no evidence that the shRNAs used are completely specific.

5. Is the localization to IR-induced foci specific to S/G2 cells as would be expected if it requires resection of the DNA end?

Referee #3:

This paper reports the characterization of a human hortologue of the primpol enzyme originally described in bacteria. Evidence is presented that this enzyme forms a physical complex with the ssDNA binding protein RPA and that it is recruited into nuclear foci co-localizing with RPA upon treatment of cells with the DNA synthesis inhibitor hydroxyurea (HU) or following formation of DNA double strand breaks (DSBs) induced by ionizing irradiation (IR). Moreover, biochemical experiments are presented showing that recombinant primpol displays primase and DNA polymerase activity in vitro. Furthermore evidence is presented showing that primpol is required for restart of replication forks arrested with HU and that the catalytic activity of primpol is required for this function. Finally, it is shown that cells depleted of primpol are sensitive to both HU and IR.

This report is interesting and important since describes the first characterization of primpol in eukaryotic cells. The experiments are well performed and the results are clear cut. I therefore reckon that this works merits publication in EMBO Reports after the points described below will be entirely satisfied.

1. The characterization of the primpol antibody should be presented since this is the first primpol antibody to be described in eukaryotes. Moreover, this antibody is used to detect primpol in immunoprecipitation experiments to demonstrate interaction with RPA and in immunofluorescence (IF). The reactivity of preimmune and immune sera should be presented on SDS-PAGE loaded with total cell extracts, and the observed molecular mass must be determined. In addition, the specificity of the IF staining with this antibody must be controlled by showing the staining in cells treated with primpol shRNA in the presence of HU or IR.
2. In figure 1I foci of hprimpol ectopically expressed are shown. It is important to show whether endogenous hprimpol also forms foci in cells treated with HU or IR.
3. It is claimed that the RPA-primpol interaction is increases upon induction of replication arrest with HU, however in Figure 1F-G there's no indication that this is the case. This point should be further demonstrated, otherwise this claim must be withdrawn.
4. One major claim of this paper is that downregulation of primpol by shRNA leads to spontaneous DNA damage. This claim is drawn upon observation of gamma-H2AX foci or chromosome abnormalities. I reckon that this claim cannot be made unless more DNA damage markers are analyzed. Although gamma-H2AX is a marker of DSBs, it is well known that many other types of DNA damage, replication stress or changes in chromatin structure and/or topology can easily induce gamma-H2AX staining. Therefore as it stands, this result cannot be unambiguously used as a marker of spontaneous DNA damage. This point must be clarified in the text. The presence of the following

DNA damage markers must in parallel be determined: pCHK2; pATM; 53BP1. This information is also required to sustain the model presented in Figure 7 in which formation of DSBs in the absence of primpol is proposed.
5. The most important part of this paper is the claim that primpol is required for replication fork restart. This conclusion is drawn from experiments in which HU is added for 2 hours to stall replication forks and then withdrawn. The requirement for primpol in replication forks restart is then assessed by analysis of replication dynamics by fiber stretching following labeling of ongoing and restarted replication forks with two different fluorescent labels (red and green respectively). A quantification of the mean size of the tracks observed in cells treated with either control or primpol shRNA must be provided. Finally the size of the fibers obtained upon stretching in both conditions must be indicated. These measurements are absolutely essential in order to make the quantification data and the role of primpol in replication forks restart believable.
6. For the sake of accuracy, in the model depicted in figure 7, leading and lagging strands must be clearly pictured. I believe that most of RPA will accumulate on leading strand while on the lagging strand there may be less RPA due to the presence of replication intermediates.

Minor points:

-In Figure 5F representations of karyotypes from control or Hela cells treated with shRNA are presented. Although I am no a cytogeneticist, at a first glance it looks as if the magnification of the two panels is not the same, otherwise, the size of the chromosomes in the two conditions is different. Looking at the panels, it also seems as if the number of chromosomes are different in the two conditions. Moreover in the quantification shown in panel G, a statistical test to determine the p value is required to be confident that the differences are significatives. Probably Hela cells are not the best cells to analyze chromosome abnormalities. My suggestion is to leave these panels out and maybe keep the quantification with the addition of the p value.

-The observation that primpol is recruited to DSBs foci but that cells depleted of primpol are not hyhpersensitive to this damage, while they are sensitive to HU must be interpreted in the discussion section. In addition, a role for primpol at DSBs must be speculated.

1st Revision - authors' response 01 September 2013

Response to reviewers:

Referee #1:

Summary
1. This manuscript presents a structure-function study of a novel human protein hPrimpol1 of the archaeal-eukaryotic primase family.
2. The authors have mapped a direct physical interaction of recombinant hPrimpol1 with human RPA70 subunit in vitro, and show using appropriate controls that the two proteins localize in subnuclear DNA damage foci in human (HeLa) cells exposed to HU or ionizing radiation. They further demonstrate that hPrimpol1 has intrinsic primase and DNA polymerase activities that can be inactivated by single residue substitutions. Lastly, they show using shRNA-depleted HeLa and knockdown cells reconstituted with silencing-resistant hPrimpol1 and mutants that both the primase activity and the interaction with RPA contribute to the ability of cells to recover from replication stress in several different assays.
3. Taken together the data suggest a potentially very significant role for this protein in counteracting replication stress in cultured cells.
4. These findings are of general interest to the molecular biology community and more broadly in biological evolution. They are of special interest to genome stability and cancer researchers.

Thanks for the nice summary!

Questions/suggestions that should be addressed:
5. Fig. 5C (and 6C). The legend mentions no treatment of cells with HU or other damage-inducing agent, suggesting that depletion of hPrimpol1 was sufficient by itself to induce robust gH2AX foci in

the indicated % of the cells - correct?

Yes, it is correct.

6. Fig. 5J. A simple depletion of hPrimpol1 in lanes 2-3 was not sufficient to induce phospho-Chk1 signaling in the absence of HU. Did the authors also test phospho-Chk2? What checkpoint kinase responds in the absence of induced damage to generate gH2AX in Figure 5C and 6C, but not in 5J?

Thanks for the advice! We have now examined the level of phospho-Chk2 in hPrimpol1-depleted cells. As shown in the revised Supplementary Figure S3C, hPrimpol1-depleted cells displayed a slight spontaneous phosphorylation of the checkpoint-kinase Chk2. Moreover, hPrimpol1 depletion displays a spontaneously elevated level of pATM focus formation (Supplementary Figure S3A-B). Based on these observations, we speculate that ATM/Chk2 may be the kinase that responds in the absence of induced damage to generate gH2AX in hPrimpol1-depleted cells.

7. Fig. 7E DNA fiber images, except for the vector image, do not seem to correlate well with the bar graphs shown in 7F. The authors should provide the detailed data for each sample (number of tracts of each length measured) in a supplement to justify the bar graphs shown here. The methods on p. 21-2 provide no information on the quantification.

Thanks for the advice! The same issue was also raised by reviewer#3. We have now provided the detailed data for each sample in the revised manuscript (Please see the revised Figure 5E and Supplementary Table S1).

8. Discussion. The authors have so far not noted the existence of conserved re-priming pathways among prokaryotes and the question of whether such pathways exist among eukaryotes. The impact of their findings might be heightened by a brief mention of what is known about re-priming.

We agree with this reviewer and have now included a brief mention of re-priming in the revised manuscript.

Minor clarifications
Fig. 1 legend, panel E. What do the authors mean by Morc3?

Morc3 was used as a negative control in the Co-IP experiments.

Fig. 1, panel I legend. Do the authors mean to say that the cells were exposed to 2 mM HU for a 16 h time period and to 3 Gy IR for a 3 h time period?

The cells were treated with either 2 mM HU for 16 hr or 10 Gy IR followed by recovery for 3 hr.

Referee #2:

This article presents the identification of CCDC111 (renamed hPrimpol1 by the authors) as a replication stress response protein that localizes to stalled forks via an interaction with RPA. The authors report that purified Primpol1 has primase and DNA polymerase activity. Knockdown with shRNA causes an increase in H2AX phosphorylation and a defect in recovery from replication stress. Overall the data support the authors conclusions and the results are significant and interesting. I offer only minor comments for improvement.

Thanks for the nice summary!

1. The authors state that RPA binding to the C-terminal region of Primpol1 does not influence its enzymatic activity. Since those biochemical assays were done in the absence of RPA, the authors need to qualify their conclusions.

We agree with this reviewer and have now toned down our claims in the revised manuscript.

2. Presumably the increase in H2AX when Primpol is knocked down happens due to problems in S-phase. It would be useful to document this experimentally.

Thanks for the advice! As suggested, we have now investigated the cell cycle dependence of these gH2AX foci by co-staining with cyclin A (a marker for S/G2-phase cells) and found that more than 90% of cells with gH2AX foci were Cyclin A positive, suggesting that hPrimpol1 prevents DSBs from arising during DNA replication (Please see the revised Figure 4C).

3. The interaction of many proteins with RPA1 have been documented extensively and a consensus binding motif described (see Xu et al., MCB 2008 for example). It would be useful for the authors to examine the C-terminal domain of Primpol for this binding motif.

Thanks for the suggestion! Using sequence homology alignment, we found that the RPA1-binding region in hPrimpol1 does not match the consensus sequence as described (data not shown). The citation is now included in the revised manuscript.

4. Please remove the word "specifically" from the sentence on page 11 describing the shRNAs. It is very unlikely that any shRNAs are completely "specific" to one gene. The documentation that the effects are not off-target is sufficient but there is no evidence that the shRNAs used are completely specific.

As suggested, we have now removed the word “specifically” from the revised manuscript.

5. Is the localization to IR-induced foci specific to S/G2 cells as would be expected if it requires resection of the DNA end?

Thanks for the advice! We now examined the RPA2 and hPrimpol1 foci formation in control and CtIP-depleted cells after IR treatment (CtIP is a key factor in DNA-end resection). As shown in the revised Supplementary Figure S1C-D, CtIP depletion abolished IR-induced recruitment of RPA and the downstream hPrimpol1 to DNA damage sites.

Referee #3:

This paper reports the characterization of a human hortologue of the primpol enzyme originally described in bacteria. Evidence is presented that this enzyme forms a physical complex with the ssDNA binding protein RPA and that it is recruited into nuclear foci co-localizing with RPA upon treatment of cells with the DNA synthesis inhibitor hydroxyurea (HU) or following formation of DNA double strand breaks (DSBs) induced by ionizing irradiation (IR). Moreover, biochemical experiments are presented showing that recombinant primpol displays primase and DNA polymerase activity in vitro. Furthermore evidence is presented showing that primpol is required for restart of replication forks arrested with HU and that the catalytic activity of primpol is required for this function. Finally, it is shown that cells depleted of primpol are sensitive to both HU and IR.

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1. The characterization of the primpol antibody should be presented since this is the first primpol antibody to be described in eukaryotes. Moreover, this antibody is used to detect primpol in immunoprecipitation experiments to demonstrate interaction with RPA and in immunofluorescence (IF). The reactivity of preimmune and immune sera should be presented on SDS-PAGE loaded with total cell extracts, and the observed molecular mass must be determined. In addition, the specificity of the IF staining with this antibody must be controlled by showing the staining in cells treated with primpol shRNA in the presence of HU or IR.

As suggested by the reviewer, we have now characterized the hPrimpol1 antibody (Please see the revised Supplementary Figure S1A-B). Unfortunately, while we have generated several hPrimpol1 antibodies, none of them is suitable for immunofluorescence staining.

2. In figure 1I foci of hprimpol ectopically expressed are shown. It is important to show whether endogenous hprimpol also forms foci in cells treated with HU or IR.

We agree with the reviewer that it is important to show whether endogenous hPrimpol1 also forms foci in cells treated with HU or IR. Unfortunately, while we have generated several hPrimpol1 antibodies, none of them is suitable for immunofluorescence staining.

3. It is claimed that the RPA-primpol interaction is increases upon induction of replication arrest with HU, however in Figure 1F-G there's no indication that this is the case. This point should be further demonstrated, otherwise this claim must be withdrawn.

The reviewer may have misunderstood our original claim: “the hPrimpol1-RPA complex formation was DNA damage independent”.

4. One major claim of this paper is that downregulation of primpol by shRNA leads to spontaneous DNA damage. This claim is drawn upon observation of gamma-H2AX foci or chromosome abnormalities. I reckon that this claim cannot be made unless more DNA damage markers are analyzed. Although gamma-H2AX is a marker of DSBs, it is well known that many other types of DNA damage, replication stress or changes in chromatin structure and/or topology can easily induce gamma-H2AX staining. Therefore as it stands, this result cannot be unambiguously used as a marker of spontaneous DNA damage. This point must be clarified in the text. The presence of the following DNA damage markers must in parallel be determined: pCHK2; pATM; 53BP1. This information is also required to sustain the model presented in Figure 7 in which formation of DSBs in the absence of primpol is proposed.

Thanks for the suggestion! We have now examined the level of phospho-CHK2 in hPrimpol1-depleted cells. As shown in Supplementary Figure S3C, hPrimpol1-depleted cells displayed a slight spontaneous phosphorylation of CHK2. Moreover, hPrimpol1 depletion displays a spontaneously elevated level of pATM and 53BP1 foci formation (Supplementary Figure S3A-B). Together, these data support our previous hypothesis that down-regulation of hPrimpol1 leads to spontaneous DSBs.

5. The most important part of this paper is the claim that primpol is required for replication fork restart. This conclusion is drawn from experiments in which HU is added for 2 hours to stall replication forks and then withdrawn. The requirement for primpol in replication forks restart is then assessed by analysis of replication dynamics by fiber stretching following labeling of ongoing and restarted replication forks with two different fluorescent labels (red and green respectively). A quantification of the mean size of the tracks observed in cells treated with either control or primpol shRNA must be provided. Finally the size of the fibers obtained upon stretching in both conditions must be indicated. These measurements are absolutely essential in order to make the quantification data and the role of primpol in replication forks restart believable.

Thanks for the advice! The same issue was also raised by reviewer#1. We have now provided the detailed data for each sample in the revised manuscript (Please see the revised Figure 4G-H, Figure 5E, Supplementary Figure S3D, and Supplementary Table S1).

6. For the sake of accuracy, in the model depicted in figure 7, leading and lagging strands must be clearly pictured. I believe that most of RPA will accumulate on leading strand while on the lagging strand there may be less RPA due to the presence of replication intermediates.

Thanks for the suggestion! The leading and lagging strands are now indicated in the revised Figure 5G. Also, we agree with the reviewer that most of RPA will accumulate on leading strand while on the lagging strand there may be less RPA due to the presence of replication intermediates. We have now changed the model accordingly.

Minor points:

-In Figure 5F representations of karyotypes from control or Hela cells treated with shRNA are presented. Although I am no a cytogeneticist, at a first glance it looks as if the magnification of the two panels is not the same, otherwise, the size of the chromosomes in the two conditions is different. Looking at the panels, it also seems as if the number of chromosomes are different in the two conditions. Moreover in the quantification shown in panel G, a statistical test to determine the p value is required to be confident that the differences are significatives. Probably Hela cells are not the best cells to analyze chromosome abnormalities. My suggestion is to leave these panels out and maybe keep the quantification with the addition of the p value.

According to the reviewer's suggestion, we have now removed the original Figure 5F in the revised manuscript. Moreover, we have now included the p value in the revised Figure 4E.

-The observation that primpol is recruited to DSBs foci but that cells depleted of primpol are not hyhpersensitive to this damage, while they are sensitive to HU must be interpreted in the discussion section. In addition, a role for primpol at DSBs must be speculated.

Thanks for the advice! Given that hPrimpol1-depleted cells show little or no hypersensitivity to IR, we speculate that hPrimpol1 may only play a mild role in the repair of IR-induced DNA damage, although it can be recruited to IR-induced DNA damage sites. We have now included this information in the revised manuscript.

2nd Editorial Decision 16 Septermber 2013

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. Referee 3 still has a few minor suggestions that I would like you to address in the manuscript text before we can proceed with the official acceptance of your manuscript.

I also noticed that scale bars are missing from all the figure panels with microscope images (both in the main and supplementary figures). Can you please add these and specify their length in the figure legends?

I finally would like to suggest some minor changes to the title and abstract as follows:

hPrimpol1/CCDC111 is a human DNA primase-polymerase that protects stalled replication forks

Prim-pol is a recently identified DNA primase-polymerase belonging to the Archaeao-eukaryotic Primase (AEP) superfamily. Here, we characterize a previously unrecognized prim-pol in human cells, which we designate hPrimpol1 (human primase-polymerase 1). hPrimpol1 possesses primase and DNA polymerase activities in vitro, interacts directly with RPA1 and is recruited to sites of DNA damage and stalled replication forks in an RPA1-dependent manner. Cells depleted of hPrimpol1 display increased spontaneous DNA damage and defects in the restart of stalled replication forks. Both RPA1-binding and the primase activity of hPrimpol1 are required for its cellular function during DNA replication. Our results indicate that hPrimpol1 is a novel factor involved in the response to DNA replication stress.

Can you please let me know whether you agree with these changes?

I look forward to seeing a new revised version of your manuscript as soon as possible.

Referee #2:

The authors satisfied all my concerns. I recommend publication.

Referee #3:

I am pleased to see that the authors have adequately satisfied the criticisms that I have raised. Meanwhile, there are still some minor concerns that need to be clarified, as described below.

Point 1. Authors need to clearly state that the Primpol antibody recognizes a doublet at the expected MW plus three non-specific polypeptides (to be highlighted with asterisks), the first showing an electrophoretic mobility of 100 kDa, the second of about 80-85(?) kDa and the third of about 55 kDa. Although shRNA-bsed experiments clearly shows that this antibody recognizes Primpol, I am actually surprised to see that the affinity-purified antibody still recognizes two non-specific bands present in the pre-immune serum, suggesting that the purification was not much effective.

Point 4. (See also point 5 raised by referee 2). The authors now show that γH2AX foci formation in primpol depleted cells is a likely result of ATM activation following DSBs formation. In addition authors now also provide new data showing that this damage occurs in S-phase. This is expected to also increase the PChk1 level as a result of ATR recruitment since the authors now show that in primpol depleted cells DSBs requires CtIP, indicating that resection occurs. Actually, by carefully looking at the PChk1 signal shown in Figure 5I, lane 3, there may be a significant increase in PChk1 in cells depleted of primpol, to a similar extent than PChk2 now shown in supplementary Figure S3. Perhaps the authors can quantify this signal, normalized to the loading control, and do the same with the PChk2 signal shown in Supplementary Figure S3 to see whether the increase is significative. Notwithstanding, the authors must comment on this issue.

Page 10, last sentence. I suggest to change "essential" to "important" since upon depletion of primpol the extent of DNA damage does not seem to be elevated (as assessed by 53BP1 foci quantification), and moreover, no clear data showing its "essential" function in maintenance of genome stability have been provided in this work.

2nd Revision - authors' response 17 September 2013

Referee #2:

The authors satisfied all my concerns. I recommend publication.

Thanks!

Referee #3:

I am pleased to see that the authors have adequately satisfied the criticisms that I have raised.

Thanks!

Meanwhile, there are still some minor concerns that need to be clarified, as described below.
Point 1. Authors need to clearly state that the Primpol antibody recognizes a doublet at the expected MW plus three non-specific polypeptides (to be highlighted with asterisks), the first showing an electrophoretic mobility of 100 kDa, the second of about 80-85(?) kDa and the third of about 55 kDa. Although shRNA-bsed experiments clearly shows that this antibody recognizes Primpol, I am actually surprised to see that the affinity-purified antibody still recognizes two non-specific bands present in the pre-immune serum, suggesting that the purification was not much effective.

We agree with this reviewer and also noted that the anti-hPrimpol1 antibody recognized a doublet by Western blot, which may suggest that hPrimpol1 is post-translationally modified. We have now included this information in the Supplementary Information.

Point 4. (See also point 5 raised by referee 2). The authors now show that γH2AX foci formation in primpol depleted cells is a likely result of ATM activation following DSBs formation. In addition authors now also provide new data showing that this damage occurs in S-phase. This is expected to also increase the PChk1 level as a result of ATR recruitment since the authors now show that in

primpol depleted cells DSBs requires CtIP, indicating that resection occurs. Actually, by carefully looking at the PChk1 signal shown in Figure 5I, lane 3, there may be a significant increase in PChk1 in cells depleted of primpol, to a similar extent than PChk2 now shown in supplementary Figure S3. Perhaps the authors can quantify this signal, normalized to the loading control, and do the same with the PChk2 signal shown in Supplementary Figure S3 to see whether the increase is significative. Notwithstanding, the authors must comment on this issue.

Thanks for the suggestion. Actually, we have repeated this experiment several times and found that hPrimpol1 depletion had no discernible effect on CHK1 phosphorylation.

Page 10, last sentence. I suggest to change "essential" to "important" since upon depletion of primpol the extent of DNA damage does not seem to be elevated (as assessed by 53BP1 foci quantification), and moreover, no clear data showing its "essential" function in maintenance of genome stability have been provided in this work.

Thank you for your suggestions! We have changed the statement in the revised manuscript.

