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ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery

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(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 30 April 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. I am pleased to inform you that all of these referees consider your findings on the interplay of ATR, ATM and WRN helicase at stalled replication forks and during their recovery interesting and thus potentially well-suited for publication in The EMBO Journal. They nevertheless raise a number of substantive issues that would need to be satisfactorily addressed before publication may ultimately be warranted. As you will see, the majority of these criticisms pertain to technical issues related to the presented data, their quality and their conclusiveness, and I would therefore like to give you the opportunity to address these concerns in the form of a revised manuscript. Should you be able to adequately improve these aspects criticized by the reviewers, we should be able to consider the study further for publication. In this respect, I should however point out that this may likely necessitate the repetition of a number of experiments to obtain more definitive results, and please also take the concerns regarding image quality, contrast settings etc. seriously (please see also the information on image processing below and in our guide to authors). In addition to the various technical and discussion points, there is nevertheless also one conceptual concern voiced by both reviewers 2 and 3 that would need to be improved, namely a better separation of ATR- and ATM-dependent effects on WRN e.g. by selective mutation of specific S/TQ target sites (see referee 2 point 1, and referee 3 point 4).

I should remind you that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case,

please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript from Pichierri and colleagues addresses important issues regarding the interplay of the WRN helicase and the checkpoint kinases ATR and ATM at stalled replication forks in human cells. This work follows on earlier studies from the same lab showing that WRN plays a central role in the cellular response to replication stress. Here, the authors provide evidence that WRN is phosphorylated by ATR in vitro and in vivo and that mutation of 6 S/TQ sites in the C-terminal region of WRN (WRN6A) severely impairs cells ability of recover from a hydroxyurea arrest, to the same extent as ATR-deficient cells. Moreover, they show that expression of the WRN6A leads to a more severe phenotype than the absence of WRN, indicating that this mutant acts in a dominant negative manner on other fork recovery pathways. Interestingly, they also uncover a second level of regulation of WRN that depends on ATM. These data are integrated in a model stipulating that WRN is first phosphorylated by ATR to facilitate its maintenance at arrested forks and prevent fork collapse. In case of prolonged fork arrest, WRN is then phosphorylated by ATM to displace it from DNA double strand breaks (DSBs) and allow the recombinational repair of DSBs by Rad51.
Overall, the manuscript is sound and well written. The model proposed by the authors is well supported by the data and should have a major impact on the DNA replication and genome integrity fields. However, several important issues should be addressed, which are listed below:

Major issues:

1) The quality of some of the gels is sometimes not sufficient to support the conclusions drawn by the authors and should be improved. For instance, the contrast of the WB shown in Fig.1D is too high to discriminate between the beta and gamma forms. It is also difficult to appreciate the extent of ATR depletion in Fig.3C. This is a problem as this information is essential to support the view that the effect of WRN6A is as severe as ATR depletion.
2) Figure 2D is missing.
3) The DNA fiber images shown in Fig.4D are not convincing. Panel (a) shows overlapping green and red signals resembling more initiation events than restarting forks. The authors should show a representative field of view with restarting and collapsed forks for all cell lines, as in Peterman, 2010.

Minor issues:

1) Panel 1A is too small.
2) Panel 2B could be replaced with Sup. Fig. 1
3) Viability is expressed as a percentage of dead cells in Fig. 5D and as a percentage of viable cells in the corresponding text. This should be homogenized for clarity.
4) Are differences shown in Fig. 6C statistically significant?
5) Three different spelling are used for the ATM-dead WRN mutant in fig7.

Referee #2 (Remarks to the Author):

Werner syndrome protein (WRN) promotes genome stability by functioning in several DNA repair pathways. Recent studies have indicated that WRN plays an important role in reactivation of stalled replication forks. The manuscript by Ammazzalorso et al. addresses the functional role of ATR- and ATM-dependent phosphorylation of WRN occurring in response to replication arrest. The authors demonstrated that phosphorylation of WRN early after replication arrest was mainly dependent on ATR. Moreover they found that ATR phosphorylated WRN directly, and that alanine substitutions at six potential ATR/ATM target sites (S/TQ) clustered at the C-terminus of WRN completely abolished ATR-mediated phosphorylation of WRN in vitro and in vivo. Detailed phenotypic analyses revealed that this unphosphorylable mutant of WRN (WRN6A) failed to properly localize to stalled replication forks to promote replication restart and accumulated at sites of DNA double-strand breaks resulting from fork collapse. Cells expressing the WRN6A mutant exhibited even higher degree of fork breakage than WRN-deficient cells due to a defect in the activation of RAD51-dependent repair pathway. The authors also characterized a WRN mutant that was resistant only to ATM phosphorylation. Like WRN6A, this mutant showed a prolonged persistence in nuclear foci during recovery from replication arrest and inhibited the formation of RAD51 foci. Similar phenotypes were also observed upon inactivation of ATM in WRN-proficient cells. Based on these findings, the authors propose that ATR phosphorylates WRN in response to stalled forks to prevent fork collapse, whereas ATM promotes delocalization of WRN from collapsed forks to allow for RAD51-mediated replication recovery.
In sum, this work brings significant insight into the molecular mechanisms involved in the cellular response to replication arrest. However, there are some issues that should be addressed before publication.

Specific Comments:

1. The conclusion that ATR phosphorylation of WRN is required for efficient re-localization of WRN to stalled replication forks and for suppression of their breakage is based on data from phenotypic analysis of a mutant that is defective for both ATR and ATM phosphorylation. Since it is also stated that ATR and ATM phosphorylate WRN at different sites, it is not clear why the authors did not precisely map the sites of ATR phosphorylation on WRN and use appropriate mutant in their study. I think that the data obtained with the WRN6A mutant do not allow authors to draw conclusions regarding the role of ATR phosphorylation in WRN function. In order to identify ATR sites on WRN, the C-terminal fragment of WRN phosphorylated by immunoprecipitated ATR should be subjected to mass spectrometric analysis. Alternatively, different combinations of point mutations in the C-terminal S/TQ cluster should be generated and the resulting mutants subjected to in vitro kinase assays with both ATR and ATM.

2. The manuscript would benefit if Figure 1D and the corresponding text were omitted - "gamma" form of Myc-WRNdeltaN is not clearly apparent. In addition, there is no clear difference between treated and non-treated cells in the level of Myc-WRNdeltaN phosphorylation.

3. Figure 2D is missing in the manuscript.

4.The anti-pS/TQ blot shown in Figure 6B is of poor quality and does not support the statements in the text on page 12 - no significant decrease in WRN phosphorylation is apparent after addition of ATM inhibitor to cells recovering from replication arrest. Moreover, it is surprising that the level of WRN phosphorylation detected in non-treated cells is even higher than that in HU-treated cells.

5. Supplementary Figure 4B: Graph showing percentage of gamma-H2AX nuclei should be added.

6. Scale bars should be added to all Figures with immunofluorescence images (including Fig. 4D)

Minor Comments:

1. Page 7, line 6: change Figure 1A to Figure 2A.

2. Page 12, line 12: refer to Supplementary Figure 4A and B.

2. Figure 5D: change WT to WSWRN.

3. Figure 6A: indicate the antibody (anti-WRN) used for IF staining

4. Figure 7A: indicate the antibody (anti-RAD51) used for IF staining.

5. Figure 7C: change WT to WSWRN and WRNATdsiA+siR51 to WRNATdsiATR+siR51.

Referee #3 (Remarks to the Author):

This study investigates the role of ATR and ATM phosphorylation in the regulation of WRN function during DNA replication. In particular, the authors show that WRN is directly phosphorylated by ATR mainly at two canonical S-TQ sites located in the C-terminus of the protein. This ATR dependent phosphorylation event is important for the proper accumulation of WRN nuclear foci, colocalization with RPA, and to avoid DSB formation at stalled replication forks. Conversely, ATM phosphorylation promotes the delocalization of WRN from collapsed forks which is required for the efficient recruitment of RAD51 and the consequent activation of the RAD51-dependent recombination pathway of fork recovery. The results are interesting and point to distinct roles of the ATR and ATM kinases in preventing breakage of stalled replication forks and in promoting replication fork recovery upon fork collapse through WRN phosphorylation. Overall the data are of high quality and make an interesting addition to our knowledge of these factors. However, the significance of the paper and its suitability for EMBO J would be greatly strengthened if the authors could provide an answer to the issues listed below.

Major points:

1. The quality of the immunofluorescence experiments of Figure 2A should be improved. The authors state that formation of WRN foci was greatly reduced in WSWRN6A cells after HU-induced replication fork stalling and the graph of Figure 2B supports this conclusion. However, this decrease in foci formation is not visible in the immunofluorescence data of Figure 2B.

2. The authors state on pg. 7 that the nuclear foci formed by the mutant form of WRN (which is presumably the WSWRN6A mutant) preferentially co-localize with the DSB-marker -H2AX suggesting that "they might pinpoint spontaneous DSBs or collapsed replication forks". First, Figure 2D which should contain these data is missing in the current version of the manuscript. Second, this conclusion is not consistent with the data of Figure 2B where the authors show that the WSWRN6A cells fails to relocalize the mutant WRN in nuclear foci, at least early after replication arrest.

3. The authors state that -H2AX is a marker of DSBs and support this conclusion by comet assays showing that HU treatment promotes the formation of DSBs in WS cells. However, the fact that HU treatment results in fewer RAD51 foci compared to -H2AX foci in WS cells suggests that -H2AX might be also detecting additional forms of DNA lesions which are not necessarily DSBs.

4. The fiber track analysis of Figure 4C-E indicate that WRN phosphorylation is required for the efficient recovery from perturbed DNA replication both at early and later times after HU treatment. The authors need to show if this recovery depends upon ATR phosphorylation only by including additional control experiments using the ATM inhibitor or possibly by using a different mutant that carries the S991 and S1256 substitutions only (which according to the authors' findings are the preferred ATR substrates).

5. The data of Figure 5B showing that the amount of DSBs in WSWRN6A cells is equivalent to that of WS cells (even before release) are not consistent with the data of Figure 3A where the expression of WSWRN6A leads to an increased load of DSBs than that observed in the parental WS cells. The authors need to provide an explanation for this discrepancy.

6. The data on the ATM phosphorylation of Figure 6B are not convincing. Further studies to check this are essential.

7. Other human RecQ helicases, such as BLM, are phosphorylated by ATR and ATM, and play important roles in maintaining the stability of stalled replication forks and in promoting replication forks recovery. The paper would be strengthened if the authors could add a paragraph in the

Discussion to examine the possible similarities and/or differences between the proposed roles of the different human RecQ helicases during DNA replication.

Minor points:

1. Pg. 7, lane 6: "Figure 1A" should be corrected with "Figure 2A.

2. Pg. 11. The data on the colocalization of WRN foci with -H2AX in WSWRN6A cells should be included in the manuscript.

3. Pg. 12: the sentence "...which can be phosphorylated by ATR but not by ATM" should be corrected with "...which can be phosphorylated by ATM but not by ATR",

4. Supplementary Figure 4B should also include the data obtained with the WSWRN6A cells for a more proper comparison of WSWRN6A and WSWRNATMdead phenotypes.

1st Revision - authors' response 16 July 2010

Reviewer #1

We would like to thank this reviewer for his/her appreciation of our work. We found his/her suggestions constructive and useful to improve the quality of our work. We included all the relevant changes to clarify the points raised.

Major issues:
1) The quality of some of the gels is sometimes not sufficient to support the conclusions drawn by the authors and should be improved. For instance, the contrast of the WB shown in Fig.1D is too high to discriminate between the beta and gamma forms. It is also difficult to appreciate the extent of ATR depletion in Fig.3C. This is a problem as this information is essential to support the view that the effect of WRN6A is as severe as ATR depletion.

Our reply:
The blot previously presented in Figure 1D has been now moved to Supplementary Figure 1 also taking into account the comment of reviewer #2. However, to improve the quality of the gel, as requested by this reviewer, we repeated the experiment using a technique (Phos-tag SDS-PAGE) that results in a better resolution of the phosphorylated isoforms by electrophoresis. In addition, we performed the new experiments transfecting 293T cells instead of HeLa to increase the number of transfected cells as well as the amount of expressed protein. The new blot confirms that the C-terminal region of WRN undergoes to multiple phosphorylation in vivo. In contrast to what observed in HeLa cells, WRN N appears to be present as two phosphorylated species already in the absence of treatment in 293T cells. This result is very reproducible and the apparent discrepancy with what previously reported using HeLa cells is probably attributable to a higher load of DNA damage or replication stress in 293T cells. For what concerns Figure 3C, we have improved the quality of the gel as requested by this reviewer.

2) Figure 2D is missing.

Our reply:
We apologize for the mistake probably occurred during final assembly of the manuscript. We have now included the missing figure as Supplementary Figure 2.

3) The DNA fiber images shown in Fig.4D are not convincing. Panel (a) shows overlapping green and red signals resembling more initiation events than restarting forks. The authors should show a representative field of view with restarting and collapsed forks for all cell lines, as in Peterman, 2010.

Our reply:
We have now included new images showing, for each cell line, a representative field corresponding to the restart of stalled forks after 16h of HU, that is the experimental point at which we observe the more relevant impairment of fork restart in WRN6A. We have now included additional results from experiments performed using the "pure" ATR-unphosphorylable WRN allele WRN3A, taking into account also comments of reviewer #3.

Minor comments:
1) Panel 1A has been enlarged accordingly;
2) Panel 2B has been replaced with Supplementary Figure 1 representing sensitivity of the WRN mutants to HU and CPT. The panel showing the results of the IF analysis has been amended according to comments made by the two other reviewers and has been renamed as panel 2D;
3) Labeling of the Y-axis and the text have been corrected and now refer to as "percentage of dead cells";
4) The differences in the number of WRN-foci positive nuclei observed during the recovery from prolonged HU treatment, when ATM is inhibited or the WRNATMdead protein is expressed, are significant if compared with the data obtained in the siCTRL or siATR-treated cells;
5) We have now used the same spelling for cells expressing the ATM-unphosphorylable mutant throughout Figure 7.

Reviewer #2
We appreciated very much that this reviewer found interesting and potentially important for the field our work. We have now included additional experiments to address the reviewer's concerns on the nature of ATR phosphorylation sites. We hope that this reviewer can find interesting these new data and useful to clarify the points raised.

Specific comments:
1. The conclusion that ATR phosphorylation of WRN is required for efficient re-localization of WRN to stalled replication forks and for suppression of their breakage is based on data from phenotypic analysis of a mutant that is defective for both ATR and ATM phosphorylation. Since it is also stated that ATR and ATM phosphorylate WRN at different sites, it is not clear why the authors did not precisely map the sites of ATR phosphorylation on WRN and use appropriate mutant in their study. I think that the data obtained with the WRN6A mutant do not allow authors to draw conclusions regarding the role of ATR phosphorylation in WRN function. In order to identify ATR sites on WRN, the C-terminal fragment of WRN phosphorylated by immunoprecipitated ATR should be subjected to mass spectrometric analysis. Alternatively, different combinations of point mutations in the C-terminal S/TQ cluster should be generated and the resulting mutants subjected to in vitro kinase assays with both ATR and ATM.

Our reply:
We decided to use the completely unphosphorylable WRN mutant (WRN6A) because our data on the phosphorylation of S/TQ sites of WRN obtained depleting ATR by RNAi demonstrated that phosphorylation of WRN, under the experimental conditions used to analyse subnuclear dynamics and formation of DSBs (i.e. treatment with HU for up to 8h), was completely ATR-dependent, excluding the contribution of ATM. So, having evidence that ATR did not target ATM sites, in vitro, we found the use of WRN6A equivalent to that of a "pure" ATR-phosphorylation mutants. After the observation that expression of WRN6A was more detrimental than the absence of WRN when replication arrest is sustained (i.e. more than 8h in HU) and during recovery from the block we decided to tackle the hypothesis of a contribution of ATM phosphorylation by generating a "pure" ATM-unphosphorylable WRN mutant (WRNATMdead). However, we agree with this reviewer's suggestion and found that performing additional experiments with a "pure" ATR mutant could be of benefit to our conclusions.

Accordingly, we generated two additional mutants and tested their ability as ATR and ATM substrates by in vitro kinase assays. We observed from our previous data that mutations of the two preferred ATR substrates (i.e. S991 and S1256) did not abrogate completely phosphorylation, leaving a residual level of phosphorylation that was about 1/3 of that observed in the wild-type fragment, suggesting that another residue could be targeted by ATR in the absence of S991 and S1256. Moreover, T1152 is the only additional residue that has not been reported to be phosphorylated by ATM. Thus, T1152 was a good candidate as third ATR phosphorylation site. In vitro ATR kinase assay using the M5 mutant, which contains Ala substitutions at the two preferred ATR phosphorylation sites and at T1152, now shows that the three possible ATR phosphorylation sites are indeed S991, S1256 and T1152. Conversely, the M6 mutant, which contains Ala substitutions only at the three previously-identified ATM phosphorylation sites (S1141, S1058 and S1292), can be phosphorylated by ATR equally well as the wild-type (see Figure 1C). We also performed ATM in vitro kinase assays using the M5 and M6 mutants as substrates (see Figure 1D) and these new experiments show that, as expected, the M5 mutant is efficiently phosphorylated by ATM, whereas the M6 mutant is not.
Unfortunately, we tried to do mass spec experiments, but they did not provide conclusive results. Basing on these new evidence obtained with in vitro phosphorylation of the M5 and M6 mutants, we generated the WRN3A allele, which contains Ala substitutions at S991, T1152 and S1256 and is a "pure" ATR-unphosphorylable mutant. The WRN3A mutant has been used to stably transfect WS cells and these cells used to confirm the role of ATR phosphorylation for the WRN function. These additional experiments are now included in Figures 2, 3 and 4 (DNA fiber assay). The results presented in these figures show that expression of WRN3A does not revert the characteristic hypersensitivity to replication perturbing agents of WS cells. Furthermore, our additional experiments demonstrate that expression of WRN3A leads to an impairment of the formation of WRN nuclear foci and to accumulation of DSBs similar to those observed for the WRN6A mutant. Interestingly, the DNA fiber assay shows that expression of WRN3A, even though fails to revert the defect in replication fork restart observed in WS cells, does not determine the dramatic fork inactivation associated with the WRN6A mutant. This observation is particularly striking and further supports our model whereby loss of ATR phosphorylation impacts on early events associated with fork stalling, while loss of ATM phosphorylation is relevant whenever DSBs are formed at stalled forks.

2. The manuscript would benefit if Figure 1D and the corresponding text were omitted - "gamma" form of Myc-WRNdeltaN is not clearly apparent. In addition, there is no clear difference between treated and non-treated cells in the level of Myc-WRNdeltaN phosphorylation.

Our reply:
Since the reviewer #1 asked for an improved version of the Figure 1D and because we think that the result of this experiment may support the conclusion that WRN may be phosphorylated at multiple sites of its C-terminal region, we maintained the blot but moved it to a supplementary figure (Supplementary Figure 1) to address this reviewer suggestion. The new data obtained using Phos-tag SDS-PAGE show more clearly the presence of two well defined phosphorylated species of WRN N. We noticed that, in contrast to what observed in HeLa cells, in 293T cells WRN N exists as two phosphorylated species already in the absence of treatment. This result is very reproducible and the apparent discrepancy with what previously reported in HeLa cells is probably attributable to a higher load of DNA damage or replication stress in 293T cells.

3. Figure 2D is missing in the manuscript.

Our reply:
We apologize for the mistake probably occurred during final assembly of the manuscript. We have now included the missing figure as Supplementary Figure 2;

4.The anti-pS/TQ blot shown in Figure 6B is of poor quality and does not support the statements in the text on page 12 - no significant decrease in WRN phosphorylation is apparent after addition of ATM inhibitor to cells recovering from replication arrest. Moreover, it is surprising that the level of WRN phosphorylation detected in non-treated cells is even higher than that in HU-treated cells.

Our reply:
We have included an improved version of the anti-pS/TQ blot in which the decrease in WRN phosphorylation on S/TQ residues after ATM inhibition is more clear. Even though WRN appears phosphorylated on S/TQ residues in the absence of treatment, the revised figure shows more clearly that replication arrest and recovery increase such S/TQ phosphorylation. The presence of WRN phosphorylation in the absence of treatment is not unexpected. Indeed, we detected repeatedly anti-S/TQ immunoreactivity in WRN IP from untreated samples (see Figure 1B and 1E) and the WRN N experiments confirm that WRN exists as phosphorylated protein under unperturbed conditions.

5. Supplementary Figure 4B: Graph showing percentage of gamma-H2AX nuclei should be added.

Our reply:
We have now included a graph showing quantization of -H2AX-positive nuclei.

6. Scale bars should be added to all Figures with immunofluorescence images (including Fig. 4D)

Our reply:
We have now included scale bars to all the relevant figures.

Minor comments:
We have amended text and figures accordingly to this reviewer's suggestions and comments.

Reviewer #3

We would like to thank this reviewer for his/her appreciation of our work and for the insightful comments and suggestions. We included in the revised manuscript additional experiments that we hope will be helpful in clarifying the points raised by this reviewer.

Major points:
1. The quality of the immunofluorescence experiments of Figure 2A should be improved. The authors state that formation of WRN foci was greatly reduced in WSWRN6A cells after HU-induced replication fork stalling and the graph of Figure 2B supports this conclusion. However, this decrease in foci formation is not visible in the immunofluorescence data of Figure 2B.

Our reply:
We have included new IF images showing more clearly the decrease in the focal localization of the WRN6A mutant. According to criticisms raised by this reviewer and reviewer #2, we have also included additional data on the relocalisation in nuclear foci of a new WRN phosphorylation mutant, WRN3A, which contains Ala substitutions only at sites found phosphorylated by ATR by our previous and additional in vitro assays.

2. The authors state on pg. 7 that the nuclear foci formed by the mutant form of WRN (which is presumably the WSWRN6A mutant) preferentially co-localize with the DSB-marker -H2AX suggesting that "they might pinpoint spontaneous DSBs or collapsed replication forks". First, Figure 2D which should contain these data is missing in the current version of the manuscript. Second, this conclusion is not consistent with the data of Figure 2B where the authors show that the WSWRN6A cells fails to relocalize the mutant WRN in nuclear foci, at least early after replication arrest.

Our reply:
We apologize for the missing Figure 2D, which has been probably lost during manuscript assembly. We have now included this figure as Supplementary Figure 2. For what concerns WRN/H2AX

colocalisation in WSWRN6A cells, we found that the WRN6A mutant shows a strong reduction of its ability to relocalise in nuclear foci (as stated in the text), but is still able to form foci (see Figure 2D), even at early time-points after treatment. We amended the text accordingly.

Thus, being WRN6A-foci positive nuclei detectable, analysis of colocalisation between the WRN mutant and phosphorylated H2AX is possible. Indeed, our data suggest that both the WRN6A foci formed 8h after treatment and those formed after prolonged replication inhibition colocalise almost completely with the phospho-H2AX foci.

3. The authors state that -H2AX is a marker of DSBs and support this conclusion by comet assays showing that HU treatment promotes the formation of DSBs in WS cells. However, the fact that HU treatment results in fewer RAD51 foci compared to -H2AX foci in WS cells suggests that -H2AX might be also detecting additional forms of DNA lesions which are not necessarily DSBs.

Our reply:
The reviewer's comment is appropriate. Indeed, phospho-H2AX is an absolute DSB marker essentially after IR, when correlates pretty well with the number of DSBs evaluated with other methods. After HU treatment phospho-H2AX does not pinpoint DSBs only, but may also reflect checkpoint activation. For this reason, we performed the analysis of DSB formation also using an independent assay that directly evaluates such a kind of DNA damage (i.e neutral comet assay). We found that the two independent assays basically give similar conclusions making us confident about accumulation of DSBs in WS cells and our phospho-mutants. However, it is likely that phospho-H2AX staining may also reflects checkpoint activation thus explaining the higher number of phospho-H2AX-positive nuclei compared with those positive to RAD51-foci.

4. The fiber track analysis of Figure 4C-E indicate that WRN phosphorylation is required for the efficient recovery from perturbed DNA replication both at early and later times after HU treatment. The authors need to show if this recovery depends upon ATR phosphorylation only by including additional control experiments using the ATM inhibitor or possibly by using a different mutant that carries the S991 and S1256 substitutions only (which according to the authors' findings are the preferred ATR substrates).

Our reply:
Also according to the reviewer #2's criticisms, we now included the analysis of replication restart by DNA fiber dual labeling using the "pure" ATR-unphosphorylable WRN mutant (WRN3A). Using this WRN phospho-mutant, it appears evident that loss of ATR phosphorylation is sufficient to determine a defect in replication restart after 8h of HU, which is similar to that observed in WS cells or in the WRN6A mutant. In contrast, WRN3A does not determine the striking inactivation of stalled forks correlated with WRN6A expression. These new experiments demonstrate that ATR phosphorylation of WRN protects from DSBs at stalled forks (see Figure 3) but, once DSBs are formed, the WRN response (and fork restart as well) becomes regulated through ATM-dependent phosphorylation.

5. The data of Figure 5B showing that the amount of DSBs in WSWRN6A cells is equivalent to that of WS cells (even before release) are not consistent with the data of Figure 3A where the expression of WSWRN6A leads to an increased load of DSBs than that observed in the parental WS cells. The authors need to provide an explanation for this discrepancy.

Our reply:
In Figure 3A, we performed two types of analyses. We counted the number of phospho-H2AX-positive nuclei and we also discriminate nuclei showing a more intense staining (referred as to "high" fluorescence in the graphs). The total number of nuclei positive to phospho-H2AX immunostaining is pretty similar between WS and WSWRN6A cells in both Figure 3A and 5B. In contrast, a difference between WS and WSWRN6A cells may be observed in the fraction of phospho-H2AX-positive nuclei with high fluorescence (see graphs in Figure 3A). However, in Figure 5B we decided to show only the total number of phospho-H2AX-positive nuclei because this information was sufficient to appreciate the conclusion and for sake of clarity.

6. The data on the ATM phosphorylation of Figure 6B are not convincing. Further studies to check this are essential.

Our reply:
We have now included a new WB that more convincingly shows ATM-dependent WRN phosphorylation during recovery from prolonged HU treatment or CPT exposure.

7. Other human RecQ helicases, such as BLM, are phosphorylated by ATR and ATM, and play important roles in maintaining the stability of stalled replication forks and in promoting replication forks recovery. The paper would be strengthened if the authors could add a paragraph in the Discussion to examine the possible similarities and/or differences between the proposed roles of the different human RecQ helicases during DNA replication.

Our reply:
We included in the revised manuscript information about the relevance of ATR/ATM phosphorylation of BLM and parallelisms with WRN. We decided, however, to not include much more information on the WRN/BLM differences because of the total characters restriction.

Minor points:
We included the corrections indicated by this reviewer in the revised manuscript. We also included an additional data on WSWRN6A in the Supplementary Figure 4B (now Supplementary Figure 5B).

Acceptance letter 29 July 2010

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

You shall receive a formal letter of acceptance shortly.

Yours sincerely,

Editor
The EMBO Journal

Referee 3 (comments to authors):

The authors adequately addressed the specific comments raised in my previous letter as well as the comments of the other two reviewers. In particular, the authors included novel data using a specific ATR-unphosphorylatable WRN mutant to better discriminate the contribution of ATR and ATM phosphorylation of WRN in response to replication fork arrest. They also improved the quality of some of their results as requested by this and other reviewers. The revised manuscript is significantly improved and I recommend its acceptance for publication without further review.

