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WRNIP1 protects stalled forks from degradation and promotes fork restart after replication stress

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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 18 November 2015

Thank you again for submitting your manuscript on WRNIP1 roles at stalled replication forks to The EMBO Journal. It has now been reviewed by three expert referees, whose reports are copied below for your information. I am happy to say that all of them consider this work in principle of interest and potential importance, and we would therefore be interested in considering a revised version further for publication. Before eventual acceptance of the paper, there are however several substantial concerns, raised in particular by referees 2 and 3, that would need to be satisfactorily addressed. These include specific technical/experimental/control issues affecting the conclusiveness of some of the results, but also requests for further clarification of WRNIP1 molecular roles in protecting and restarting stalled forks. I would therefore like to invite you to address these points, together with a number of editorial and writing issues raised by the referees, through a major revision of the present study.

Since I realize that it may (within the scope of a single revision round) be difficult to address every individual point of criticism through further experiments, I would in this case invite you to send us a brief proposal (in the form of a tentative response letter) on how you might be able to answer the referees' comments; this would allow us to clarify the feasibility of the proposed revision work, and to define which improvements would be key for eventual acceptance in The EMBO Journal. We could further arrange for an extended revision period, during which time the publication of any competing work elsewhere would have no negative impact on our final assessment of your own study.

REFEREE REPORTS
Referee #1:

This paper reports interesting roles for WRNIP1, a poorly understood replication-regulatory factor, in stabilizing stalled replication forks and promoting their restart. The ATPase activity of WRNIP1 is not required for stalled fork stabilization, but plays a role in replication fork restart. Both functions are however important for genome integrity,as loss of WRNIP1 or its catalytic activity causes accumulation of DNA damage and chromosome aberrations.

After uncovering a role for WRNIP1 in stabilizing stalled replication forks from MRE11-mediated degradation using fiber assay, the authors show that WRNIP1 achieves this function in conjunction with RAD51 and BRCA2, with which it interacts. WRNIP1 defects in stabilizing stalled replication forks are epistatic with the ones of RAD51 inhibition and both RAD51 overexpression or prevention of RAD51 turnover by FBH1 knockdown can compensate for loss of WRNIP1 activity. The authors further show that WRNIP1 functions at stalled forks are important for genome integrity. This makes overall for a thorough and quite interesting study. The experiments are complex, well designed and executed, and independent lines of experimentation support individual conclusions. The model suggested by the authors is interesting and supported by the data and opens new lines of research for understanding how WRNIP1 affects RAD51 stability at stalled replication forks, FBH1 dynamics and fork restart. In my view, this study is worthy of publication in the EMBO Journal. WRNIP1/MGS1 remained mysterious in many respects till now, and this study represent an important advance in our understanding of factors that protect stalled replication forks.

The manuscript will need some further editing to improve clarity of some of the sentences. For example, "...is poor elucidated", "blocking RAD51 dismantling from chromatin fork degradation and chromosomal aberrations are attenuated in WRNIP-1 deficient cells" in the Abstract.

Referee #2:

In this manuscript, Leuzzi et al. provide a potentially interesting cell biology investigation on the role of WRNIP - a yet under-investigated protein - in the protection and restart of stalled replication forks. Using DNA fiber assays, the authors show a role for WRNIP protecting stalled forks from MRE11 degradation, which is similar and epistatic to the previously described role of BRCA2. In the absence of WRNIP, replicating chromatin displays excessive ssDNA accumulation, but reduced RAD51 loading. Furthermore, RAD51 overexpression rescues fork degradation in WRNIP-defective cells, overall suggesting that WRNIP role in fork stability entails regulation of RAD51 fork loading. An ATPase defective WRNIP mutant is comparable to WRNIP-depleted cells in terms of fork restart defects, but shows no fork degradation defect and reduced defects in additional phenotypes characterized by the authors, such as DDR activation, DNA break formation (comet assay) and chromosomal aberrations (metaphase spreads).

Overall, the manuscript includes an interesting set of in vivo observations, which certainly extend our mechanistic understanding on the role of WRNIP in the replication stress response. Most of the data are convincing and are well interpreted and described, provided that important additional experiments can be performed in revision (see below). Despite the limits outlined below, I believe that these data may eventually reach the level of broad interest and novelty required for publication in the EMBO Journal. The manuscript needs to be strengthened on the biochemical mechanisms mediating some of these phenotypes, but - once supported by additional experiments - will provide a significant advance in the understanding of the role of this protein at the replication fork.

Major points:

1) The mechanism by which WRNIP helps protecting stalled forks from extensive MRE11-dependent degradation is unclear. Is this a direct control of the nuclease or an indirect effect due to different fork loading of RAD51? The authors should assess whether chromatin recruitment and, more specifically, fork recruitment of the nuclease is different in presence and absence of WRNIP. Overall, it looks like iPOND experiments on both MRE11 and RAD51 would add significantly to

the mechanistic insight on these phenomena, as recently performed by numerous labs. Similarly, WRNIP recruitment to forks cannot be simply deduced by PLA with ssDNA (which may also accumulate at a distance from the fork, especially upon such prolonged treatments), but should be directly assessed by iPOND.

2) As the authors nicely address and show that WRNIP and BRCA2 are epistatic in fork protection, it seems essential to assess whether FBH1 depletion could rescue fork degradation also in BRCA2-defective cells, as it does in the absence of WRNIP. This may provide support to the model that effective loading and retention of RAD51 at stalled forks is the key pre-requisite to prevent massive fork degradation and is probably mediated by several proteins.

3) Neither the manuscript, nor the model provide in my view a convincing explanation on the uncoupling of "fork restart" and "fork degradation" defects observed in the WRNIP ATPase mutant. In fact, these data seem to suggest that the key role of this protein in fork restart cannot be followed by the fork-degradation read-out (the ATPase mutant is equally defective as the null, but does not show fork degradation, based on Fig. 1). This apparent discrepancy is not appropriately kept into account, while drawing important conclusions from data uniquely obtained with fork degradation assays.

4) The assay used in Fig. 3A cannot be unambiguously used to monitor ssDNA accumulation on reversed forks, as several other scenarios (e.g. unresolved flaps or other complex intermediates) may contribute to ssDNA detection on nascent DNA. Furthermore, even assuming to use these data to exclude ssDNA accumulation on regressed arms, this by no means excludes that reversed forks are the structure initially targeted by nucleolytic degradation, but that this processing extends behind forks to nascent DNA normally annealed to the parental strand (thus exposing parental ssDNA). In other words, the data in Fig. 3A and 3B do not provide valuable information on how fork degradation is started and completed, and should not be used to propose or exclude specific hypotheses in the model or in the discussion.

5) Most genome instability phenotypes described for WRNIP deficiency (ssDNA accumulation, DNA breaks, chromosomal abnormalities and accumulation of dead cells) are clearly visible also in the absence of HU and also in the ATPase mutant. However, as mentioned above, neither condition is associated with fork degradation. It would be quite relevant to modify the labeling protocol for DNA fiber spreading to directly assess minor effects of WRNIP deficiencies (both null and ATPase mutant) during unperturbed replication (i.e. fork rate, fork symmetry). It may also be interesting to probe the relevance of WRNIP for replication completion and ligation of synthesized fragments. These potential effects, although uncoupled from the degradation phenotype extensively characterized here, may in fact be even more relevant to propose and define a physiological role for WRNIP in replication and may also help explain why the observed effects on fork restart can be uncoupled from fork degradation. In this respect, the sentence at the end of page 12 ("Therefore, the WRNIP1-mediated fork protection function, rather than the role in restarting stalled forks, is responsible for chromosomal instability arising after fork stalling.) does not seem supported by the data.

6) The biochemical mechanism by which WRNIP helps loading RAD51 and/or preventing its unloading by FBH1 should be further investigated. These different steps should be dissected in dedicated biochemical experiments, in order to possibly support some of the specific statements included in the discussion (e.g. " WRNIP1 could be actually recruited at perturbed forks in vivo... and be essential in the RAD51 recruitment to ssDNA, as showed for BRCA2"). If the authors lack the biochemical expertise to run such experiments, the manuscript should be tuned down in such mechanistic hypotheses. Similarly, the proposal that formation of a specialized WRNIP/WRN/Poldelta complex may assist fork restart (mid page 15) should be either tested experimentally or removed from the manuscript.

7) The manuscript strictly needs revision to improve English phrasing and to include all relevant details (e.g. compounds, concentrations, timing of addition, etc.) in order to possibly reproduce the results. Much crucial information is not even included in the Supplementary material.

Minor points:

1) Fig. 3D looks to me pretty much redundant to Fig. 2B

2) I don't quite understand the conclusion stressed by the authors from the data in Fig. 4, i.e. " Therefore, RAD51 is required for avoiding nascent strand degradation under stressful conditions in the absence of WRNIP1". I believe a far more relevant conclusion from the same data, which stresses the "physiological" role of the protein, would be that "WRNIP protects stalled forks by effective loading or retention of RAD51".

3) I could not find among the references the Higgs et al., 2015 paper cited at page 16.

Referee #3:

A large body of evidence indicates that stalled replication forks are processed by exonucleases such as Mre11 and that recombination factors like BRCA2 and Rad51 play a key role in preventing excessive resection of newly-replicated DNA. This control of Mre11-mediated resection at arrested forks is critical to prevent fork collapse and genomic instability. In this manuscript, Leuzzi and colleagues report the important observation that WRNIP1, a poorly-characterized interactor of the WRN helicase, acts together with BRCA2 and Rad51 to regulate Mre11 activity at HU-arrested forks. Using a combination of single-molecule and immunofluorescence-based assays, they report that WRNIP has two important functions at arrested forks. It prevents the displacement of Rad51 from arrested forks by the Fbh1 helicase in order to protect nascent DNA from nucleolytic degradation by Mre11. Moreover, it promotes the restart of stalled forks, presumably by interacting with Pol delta. Interestingly, the fork restart function of WRNIP1 depends on its ATPase activity, unlike its fork protection function. Finally, the authors show that the fork protection function of WRNIP1 is important to prevent replication-associated DNA damage and chromosome breaks. Overall, the data are of high quality and are presented in a clear and logical manner. Different assays are used in a clever manner to bring new insights into the mechanism of WRNIP1 action at stalled forks. Considering the growing interest on these new aspects of fork processing and restart, this study should be of a wide general interest to the readers of EMBO Journal. Yet, several important issues need to be addressed prior to publication.

Specific issues:

1) The experiments presented in this manuscript are generally well designed. However, a few important controls need to be shown and missing information should be provided. These include the percentage of transfected cells with WRNIP1 constructs and the effect of WRNIP1 depletion/complementation on the fraction of cells in S phase. Moreover, the authors should show how the original MRC5 cells compare to MRC5 cells expressing the shRNA against WRNIP1 and complemented with the wt protein, in terms of fork progression and arrest in the presence or the absence of HU.

2) The DNA fiber experiments are convincing, but the authors should have used an antibody against ssDNA to label DNA fibers and show that terminally-arrested forks (e.g. in Fig 1C) do not correspond to broken fibers. In principle, this should be less of a problem for DNA fiber spreading than for DNA combing, but this control need to be performed anyway. Moreover, the concentration of IdU and CldU has to be indicated somewhere. The authors also need to make sure that the images shown are representative of the experiments. For instance, the CldU tracks show for the untreated control in Fig1E are 2 to 3 times shorter than IdU tracks, unlike what is shown below for the track length distribution.

3) Fig 1C shows the percentage of stalled and restarting forks in WRNIP1-deficient and complemented cells. In principle, the sum of stalled and restarting forks should equal 1 for each category, but it does not seem to be the case. What do missing forks correspond to? Moreover, the expected patterns (red vs red-green) should be drawn in the same order as for the pulses for clarity (red-green and not gree-red)

4) The supplementary tables showing statistical information for each DNA fiber experiment are nice, but they should indicate the number of the corresponding figure for each dataset. Numbers are missing at the end of table S1.

5) Figure 1F should be moved to sup data as it correspond to the only experiment performed with a different cell type.

6) Figure 3 shows that HU induces a significant increase of ssDNA production in cells complemented with the WT protein, but does not lead to the shortening of IdU tracks and to fork slow down. This apparent discrepancy should be discussed by the authors.

7) Figure 3F shows an HU-dependent accumulation of RAD51 on chromatin when WRNIP1 is present. However, there is a marked difference in RAD51 levels +/- HU in these cells, unlike in shWRNIP1 cells. This difference may suggest that WRNIP1 controls the synthesis/stability of RAD51, rather than its chromatin binding. The authors should use complementary approaches, such as IF-based assays, to strengthen their point. From this regard, the PLA assay is not entirely convincing, as it depends on the presence of ssDNA, whose formation at stalled forks is prevented by RAD51. This looks to me like a circular argument.

8) Important conclusions regarding the role of Mre11 and Rad51 are based on the use of inhibitors. Considering the specificity issues associated to the use of these inhibitors, these experiments should be confirmed with siRNAs.

9) Fig5B: is the difference between numbers of PLA spots +/- HU significant?

10) Fig6A: since gamma-H2AX foci form in S phase, the authors should provide FACS profiles to confirm that the differences they see are not due to differences in cell cycle distribution.

11) The authors repeatedly refer to the use of an anti-IdU antibody. However, all antibodies used to detect IdU and CldU were actually raised against BrdU, but show a different affinity for different halogenated nucleotides. This should be indicated in the methods section.

Preliminary Response Letter to Referees 25 November 2015

We would like to thank you and the referees for your appreciation of our manuscript. We found the referees' criticisms and suggestions insightful and useful, and we feel that the revised version of the manuscript will profit from them. We also feel that we will be able to satisfactorily address all the concerns raised by the referees, in particular those from the referee #2.

In our opinion, the major concerns raised by the referee #2 and #3 are four:
1. iPOND experiments on MRE11, RAD51 and WRNIP1 proteins to demonstrate their recruitment to replication forks (ref#2: question 1);
2. Biochemical experiments to support the statement included in the discussion about the WRNIP1 fork recruitment (ref#2: question 6);
3. The formation of the WRNIP1/WRN/Poldelta complex to assist fork restart (ref#2: question 6);
4. Additional data demonstrating that WRNIP1 regulates RAD51 chromatin binding rather than synthesis/stability (ref#3: question 7).

Our experimental strategy:
1. and **2.** The most comparable technology to iPOND assay suggested by the referee #2 is the immunoprecipitation of nascent DNA protein complexes with antibodies to halogenated nucleoside analogs (i.e. IdU or CldU), which has been extensively used to investigate recruitment of proteins, such as RAD51, to replication forks (Bryant et al., EMBO J 2009; 28: 2601–2615; Petermann et al., Mol Cell. 2010; 37:492–502; Soomyajit et al., NAR 2015; 43: 9835-9855). We intend to utilize this assay to provide evidence of the presence of MRE11, RAD51 and WRNIP1 at replication forks in vivo.

Basing on this technique, which we are already starting to develop in our lab, replication forks are pulled down following the CldU or IdU Co-IP protocol. In brief, thanks to CIdU or IdU labeling of

replication forks, and by performing a chromatin fractionation and immunoprecipitation (IP) using an anti-BrdU antibody to detect CIdU or IdU, this assay allows to directly identify the proteins present at replication forks. For this experiment, we propose to label newly replicated DNA with chlorodeoxyuridine (CldU) at different time periods before HU stalling, and investigate whether MRE11, RAD51 and WRNIP1 co-immunoprecipitate (co-IP) with CldU (i.e. restarted replication forks). The amount of MRE11, RAD51 and WRNIP1 present in the chromatin (DNA) fraction and co-IP with CldU will demonstrate that these proteins are present at sites of stalled replication forks. This biochemical approach will allow us to address also the concern of the referee about fork recruitment of WRNIP1 in vivo.

3. For what concerns the hypothesis of an involvement of the WRNIP1/WRN/Poldelta complex in assisting fork restart, we are going to include some experiments of DNA fiber assay, showing that WRN is able to assist WRNIP1 in restarting fork after HU stalling and to corroborate our hypothesis of a collaboration of WRNIP1 and WRN at stalled forks. We will perform an experiment using shWRNIP1 cells in which WRN has been down regulated by RNAi and labeled as in the scheme in Fig. 1B. We will revise consequently our model.

4. To confirm the results obtained with chromatin fractionation analysis of RAD51 (Fig. 3F), we will examine the RAD51 focal relocalization by immunofluorescence assay in shWRNIP1^{WT} and shWRNIP1 cells treated or not with HU. In addition, to exclude the possibility that WRNIP1 may play a role in the synthesis or stabilization of RAD51, we will carry out biochemical experiments in which, using the MG132 proteasome inhibitor, we can assess whether RAD51 undergoes degradation in the absence of WRNIP1. Altogether, these results will allow us to demonstrate that the reduced levels of RAD51 detected in the absence of WRNIP1 are due to a role for WRNIP1 in promoting RAD51 binding to the chromatin.

Regarding the remaining concerns raised by the referees, they are mainly request of data that we can deduce from the analyses already presented, such as DNA fiber assay, or control experiments not difficult to perform, such as DNA fiber assay or FACS analysis.

In conclusion, we are confident that in the revised version of the manuscript all the referees' concerns will be satisfactorily addressed.

Editor Response to Preliminary Letter to Referees

Thank you for sending your proposal on how you intend to address the referee comments on your recent EMBO J submission. I have now had a chance to look through it and I am pleased to hear that you appear to be in a good position to answer the key queries of the reviewers. Regarding points 1 & 2, I feel that the iPOND methodology suggested by referee 2 would likely be more definitive than the CldU-IP strategy developed by Helleday and colleagues, but I understand that careful establishment and interpretation of data obtained through this alternative technique may well be able to address the referee's question equally well. In any case, should it for some reason take you longer than expected to develop these assays, feel free to contact me to discuss possible extension of the resubmission deadline!

In conclusion, I am hopeful that successful revision along the lines proposed in your letter should be able to convince the referees, and I look forward to hearing from you in due time and to reading your revision.

1st Revision - authors' response 29 February 2016

Response to Editor

We would like to thank very much you and the referees for your appreciation of our work. We found the referees' criticisms and suggestions insightful and useful, and we feel that the revised version of the manuscript has benefited from them. In the last months, we worked hard and now, we feel to have satisfactorily addressed all the concerns raised by the referees, in particular those from referees #2 and #3.

As anticipated in our previous tentative response letter to referees, in our opinion, the major concerns raised by the referee #2 and #3 were four:

1. iPOND experiments on MRE11, RAD51 and WRNIP1 proteins to demonstrate their recruitment to replication forks (ref#2: question 1);
2. Biochemical experiments to support the statement included in the discussion about the WRNIP1 fork recruitment (ref#2: question 6);
3. The formation of the WRNIP1/WRN/Poldelta complex to assist fork restart (ref#2: question 6);
4. Additional data demonstrating that WRNIP1 regulates RAD51 chromatin binding rather than synthesis/stability (ref#3: question 7).

1. and **2**. As already anticipated, to address the points **1**. and **2**., we decided to carry out the most comparable technology to iPOND assay, the CldU-IP, to detect recruitment of RAD51, MRE11 and WRNIP1 to stalled replication forks. By immunoprecipitating nascent DNA/protein complexes, an approach extensively used elsewhere to show recruitment of different factors, including RAD51, to replication forks (Bryant et al., EMBO J 2009; 28: 2601–2615; Petermann et al., Mol Cell. 2010; 37:492–502; Soomyajit et al., NAR 2015; 43: 9835-9855), we provide now direct evidence of the presence of MRE11, RAD51 and WRNIP1 at replication forks in vivo, under our experimental conditions.
3. For what concerns the hypothesis of an involvement of the WRNIP1/WRN/Polδ complex in assisting fork restart, we performed DNA fiber assay to analyze fork restart in cells depleted of WRNIP1, WRN or both. Our data show that WRN and WRNIP1 participate in a common fork restarting mechanism after HU-mediated replication arrest, corroborating our hypothesis of a collaboration of WRNIP1 and WRN at stalled forks. Given that many additional experiments have been now included in the revised version of the manuscript, we decided to not present those results for sake of clarity. However, they are provided for the reviewer's eye and we are ready to add them in the manuscript should she/he or the editors think it is useful to do so.

4. To corroborate the observation that the amount of RAD51 is lower in WRNIP1-deficient cells than in wild-type cells (Fig. 3F) that was obtained by chromatin fractionation analysis, we now included immunofluorescence analyses, which confirm the presence of a reduced RAD51 recruitment in shWRNIP1 cells. In addition, to exclude the possibility that WRNIP1 might play a role in the stabilization of RAD51, we carried out biochemical experiments using the proteasome inhibitor MG132, and provide evidence that RAD51 does not undergo degradation in the absence of WRNIP1. Moreover, we present chromatin fractionation experiments demonstrating a recovery of RAD51 chromatin association after depletion of FBH1 in WRNIP1-deficient cells (Fig 5G), further confirming that WRNIP1 is implicated in stabilizing rather than recruiting RAD51 to stalled forks.

Below you can find a point-by-point detailed answer to all the referees' criticisms.

Response to Referees

Referee #1:
This paper reports interesting roles for WRNIP1, a poorly understood replication-regulatory factor, in stabilizing stalled replication forks and promoting their restart. The ATPase activity of WRNIP1 is not required for stalled fork stabilization, but plays a role in replication fork restart. Both functions are however important for genome integrity, as loss of WRNIP1 or its catalytic activity causes accumulation of DNA damage and chromosome aberrations.

After uncovering a role for WRNIP1 in stabilizing stalled replication forks from MRE11-mediated degradation using fiber assay, the authors show that WRNIP1 achieves this function in conjunction with RAD51 and BRCA2, with which it interacts. WRNIP1 defects in stabilizing stalled replication forks are epistatic with the ones of RAD51 inhibition and both RAD51 overexpression or prevention of RAD51 turnover by FBH1 knockdown can compensate for loss of WRNIP1 activity. The authors further show that WRNIP1 functions at stalled forks are important for genome integrity. This makes overall for a thorough and quite interesting study. The experiments are complex, well designed and executed, and independent lines of experimentation support individual conclusions. The model suggested by the authors is interesting and supported by the data and opens new lines of research for understanding how WRNIP1 affects RAD51 stability at stalled replication forks, FBH1 dynamics and fork restart. In my view, this study is worthy of publication in the EMBO Journal.

WRNIP1/MGS1 remained mysterious in many respects till now, and this study represent an important advance in our understanding of factors that protect stalled replication forks.
The manuscript will need some further editing to improve clarity of some of the sentences. For example, "...is poor elucidated", "blocking RAD51 dismantling from chromatin fork degradation and chromosomal aberrations are attenuated in WRNIP-1 deficient cells" in the Abstract.

Answer to Referee #1's comments:
We thank the referee for her/his useful criticisms to our work and for her/his appreciation of the manuscript. We have revised the whole manuscript carefully, and tried to avoid any grammar or typing error to improve understanding of the text.

Referee #2:
In this manuscript, Leuzzi et al. provide a potentially interesting cell biology investigation on the role of WRNIP - a yet under-investigated protein - in the protection and restart of stalled replication forks. Using DNA fiber assays, the authors show a role for WRNIP protecting stalled forks from MRE11 degradation, which is similar and epistatic to the previously described role of BRCA2. In the absence of WRNIP, replicating chromatin displays excessive ssDNA accumulation, but reduced RAD51 loading. Furthermore, RAD51 overexpression rescues fork degradation in WRNIP-defective cells, overall suggesting that WRNIP role in fork stability entails regulation of RAD51 fork loading. An ATPase defective WRNIP mutant is comparable to WRNIP-depleted cells in terms of fork restart defects, but shows no fork degradation defect and reduced defects in additional phenotypes characterized by the authors, such as DDR activation, DNA break formation (comet assay) and chromosomal aberrations (metaphase spreads).

Overall, the manuscript includes an interesting set of in vivo observations, which certainly extend our mechanistic understanding on the role of WRNIP in the replication stress response. Most of the data are convincing and are well interpreted and described, provided that important additional experiments can be performed in revision (see below). Despite the limits outlined below, I believe that these data may eventually reach the level of broad interest and novelty required for publication in the EMBO Journal. The manuscript needs to be strengthened on the biochemical mechanisms mediating some of these phenotypes, but - once supported by additional experiments - will provide a significant advance in the understanding of the role of this protein at the replication fork.

Answer to Referee #2's comments:
We thank the referee for her/his constructive suggestions to our work and for her/his appreciation of the manuscript. We have revised the manuscript taking into account the referee's suggestions (*see below*).

Major points:
1) *The mechanism by which WRNIP helps protecting stalled forks from extensive MRE11-dependent degradation is unclear. Is this a direct control of the nuclease or an indirect effect due to different fork loading of RAD51? The authors should assess whether chromatin recruitment and, more specifically, fork recruitment of the nuclease is different in presence and absence of WRNIP. Overall, it looks like iPOND experiments on both MRE11 and RAD51 would add significantly to the mechanistic insight on these phenomena, as recently performed by numerous labs. Similarly, WRNIP recruitment to forks cannot be simply deduced by PLA with ssDNA (which may also accumulate at a distance from the fork, especially upon such prolonged treatments), but should be directly assessed by iPOND.*

According to the referee's suggestion, we have now introduced new biochemical experiments to assess chromatin recruitment of MRE11, in presence or absence of WRNIP1 (Fig. 3B).
To provide evidence of the presence of MRE11, RAD51 and WRNIP1 at stalled replication forks, we performed the chromatin fractionation and CldU-co-immunoprecipitation (Co-IP) assay. We used this method since it is considered the most comparable technology to iPOND assay (Sirbu et al., Nat Protoc. (2010) 7, 594-605), and because we were already developing it in our lab, and also because iPOND would have required more time to be set up. Although we agree that iPOND is more powerful respect of CldU-Co-IP, this technique has been extensively used in many reputed labs (Somyajit et al., NAR (2015) 43, 9835-9855; Bryant et al., EMBO J (2009) 28, 2601–2615). Using CIdU labeling of replication forks and IP using anti-BrdU antibody to detect CIdU, we show

the presence of WRNIP1, RAD51 and MRE11 at stalled replication forks (Fig. 3D). Interestingly, our analysis shows that RAD51 and MRE11 are differently recruited to stalled replication forks in WRNIP1-deficient cells. It is worth noting that our CldU-Co-IP experiments demonstrate that WRNIP1 is associated with stalled replication forks, confirming observation obtained by iPOND in a previous study from the Cortez's group (Dungrawala et al., 2015), and indirectly proving that, in our situation, CldU-Co-IP and iPOND are probably interchangeable approaches.
In view of the new data, the figure showing the association of WRNIP1 with nascent strand ssDNA by PLA (Fig. 3G in the previous version of the manuscript) has been now removed because considered unnecessary.

2) *As the authors nicely address and show that WRNIP and BRCA2 are epistatic in fork protection, it seems essential to assess whether FBH1 depletion could rescue fork degradation also in BRCA2-defective cells, as it does in the absence of WRNIP. This may provide support to the model that effective loading and retention of RAD51 at stalled forks is the key pre-requisite to prevent massive fork degradation and is probably mediated by several proteins.*

To address this interesting point, we carried out new DNA fiber experiments to analyze nascent strand degradation after depletion of BRCA2, FBH1 or both by RNAi. Not surprisingly, our results indicate that FBH1 depletion fails to rescue nascent strand degradation induced by loss of BRCA2. This new result suggests that in the absence of BRCA2, as RAD51 cannot be efficiently recruited, the presence of FBH1 is irrelevant simply because it cannot extract from chromatin anything. Moreover, this new result contributes to strengthen our hypothesis of a WRNIP1 role in stabilizing RAD51 on chromatin rather than in recruiting it. Indeed, in the absence of WRNIP1, RAD51 would undergo to unscheduled FBH1-mediated extraction from chromatin resulting in an FBH1-sensitive fork degradation phenotype as opposed to the FBH1-insensitive fork degradation phenotype associated with loss of BRCA2 (Appendix Fig S11).

3) *Neither the manuscript, nor the model provide in my view a convincing explanation on the uncoupling of "fork restart" and "fork degradation" defects observed in the WRNIP ATPase mutant. In fact, these data seem to suggest that the key role of this protein in fork restart cannot be followed by the fork-degradation read-out (the ATPase mutant is equally defective as the null, but does not show fork degradation, based on Fig. 1). This apparent discrepancy is not appropriately kept into account, while drawing important conclusions from data uniquely obtained with fork degradation assays.*

According to the referee's suggestion, we have now better explained the apparent discrepancy on the uncoupling of "fork restart" and "fork degradation" defects observed in the WRNIP1 ATPase mutant in the revised version of our manuscript. In our opinion, there is a differential requirement for WRNIP1 during the two processes. Indeed, the presence of WRNIP1 is crucial for the RAD51 stabilization at stalled forks, therefore preventing fork degradation by MRE11. From this point of view, the ATPase-dead WRNIP1 is proficient in ensuring RAD51 stabilisation, however, if abrogation of ATPase activity in the WRNIP1 mutant is not sufficient to cause fork destabilization it is enough to hamper fork restart. Indeed, in agreement with previous *in vitro* studies (Tsurimoto et al., Genes to Cells (2005) 10; 13-22), restart of stalled replication forks might require the support of the ATPase activity of WRNIP1 to stimulate DNA polymerase δ to re-initiate DNA synthesis, a possibility that needs additional investigations to be tested and that is clearly outside the scope of this revision.

4) *The assay used in Fig. 3A cannot be unambiguously used to monitor ssDNA accumulation on reversed forks, as several other scenarios (e.g. unresolved flaps or other complex intermediates) may contribute to ssDNA detection on nascent DNA. Furthermore, even assuming to use these data to exclude ssDNA accumulation on regressed arms, this by no means excludes that reversed forks are the structure initially targeted by nucleolytic degradation, but that this processing extends behind forks to nascent DNA normally annealed to the parental strand (thus exposing parental ssDNA). In other words, the data in Fig. 3A and 3B do not provide valuable information on how fork degradation is started and completed, and should not be used to propose or exclude specific hypotheses in the model or in the discussion.*

In the revised version of our manuscript, we have amended the text taking into account the issue raised by the referee. Also, we have differently organized the experiments and reconsidered the interpretation of the data. Moreover, model and discussion have been revised accordingly.

5) *Most genome instability phenotypes described for WRNIP deficiency (ssDNA accumulation, DNA breaks, chromosomal abnormalities and accumulation of dead cells) are clearly visible also in the absence of HU and also in the ATPase mutant. However, as mentioned above, neither condition is associated with fork degradation. It would be quite relevant to modify the labeling protocol for DNA fiber spreading to directly assess minor effects of WRNIP deficiencies (both null and ATPase mutant) during unperturbed replication (i.e. fork rate, fork symmetry). It may also be interesting to probe the relevance of WRNIP for replication completion and ligation of synthesized fragments. These potential effects, although uncoupled from the degradation phenotype extensively characterized here, may in fact be even more relevant to propose and define a physiological role for WRNIP in replication and may also help explain why the observed effects on fork restart can be uncoupled from fork degradation. In this respect, the sentence at the end of page 12 ("Therefore, the WRNIP1-mediated fork protection function, rather than the role in restarting stalled forks, is responsible for chromosomal instability arising after fork stalling.) does not seem supported by the data.*

In order to address the referee's concern and comment, we now presented additional data showing fork rate and fork symmetry under unperturbed replication in wild-type, WRNIP1-deficient or ATPase mutant cells (Fig. 1B-D). These analyses demonstrate that, under unperturbed growth conditions, shWRNIP1WT, shWRNIP1 and shWRNIP1T294A cells show almost identical fork velocity (Fig 1C), and that also the frequency of asymmetric replication tracks is similar in all cell lines (Fig 1D). Thus, our data would suggest that no elongation defect is triggered when WRNIP1 or its enzymatic activity was lost unless replication is stressed. Altogether, our replication analyses may indicate that the DNA damage and genome instability observed in cells depleted of WRNIP1 or expressing its ATPase-dead mutant are not correlated with a replication defect, at least at the best of the resolution of our assay. Although we find the point raised by the referee very interesting, and consistent with a proposed function of the yeast orthologue MGS1, we feel that the assessment of replication completion and ligation of synthesized fragments would require a deep and dedicated investigation, which is beyond the scope of this study. Nevertheless, we have amended the sentence indicated by the referee to provide a more balanced discussion of our results and alternative explanations of the phenotypes observed in untreated cells.

6) *The biochemical mechanism by which WRNIP helps loading RAD51 and/or preventing its unloading by FBH1 should be further investigated. These different steps should be dissected in dedicated biochemical experiments, in order to possibly support some of the specific statements included in the discussion (e.g. " WRNIP1 could be actually recruited at perturbed forks in vivo... and be essential in the RAD51 recruitment to ssDNA, as showed for BRCA2"). If the authors lack the biochemical expertise to run such experiments, the manuscript should be tuned down in such mechanistic hypotheses. Similarly, the proposal that formation of a specialized WRNIP/WRN/Poldelta complex may assist fork restart (mid page 15) should be either tested experimentally or removed from the manuscript.*

To address the interesting referee's suggestion, we provide additional data obtained from chromatin fractionation analysis and CldU-co-IP assay that substantiate our assertion about a differential fork recruitment in vivo of RAD51 and MRE11, in presence or absence of WRNIP1 (Fig 3D). Moreover, these biochemical experiments indicate that WRNIP1 is actually recruited to perturbed forks in vivo (Fig 3D).

In addition, we now included chromatin fractionation experiments showing that, in WRNIP1-deficient cells, recruitment to chromatin of RAD51 is enhanced by depletion of FBH1 (Fig 5G). These findings contribute to give mechanistic insight into how WRNIP1 contributes to stabilize RAD51 at stalled forks.

For what concerns the hypothesis of an involvement of the WRNIP1/WRN/Polδ complex in assisting fork restart, we initially proposed to the Editor some DNA fiber experiments to demonstrate a collaboration between WRN and WRNIP1 in restarting stalled forks. Because of the large amount of additional data now included in the revised version of the manuscript, we decided to

provide the reviewer with the results of these experiments but to do not include them in the manuscript. As shown in the Figure A reported below, DNA fiber analysis showed that WRN depletion (shWRNIP1WT/siWRN), or concomitant depletion for WRN and WRNIP1 (shWRNIP1WT/siWRN), enhances the percentage of stalled forks induced by HU respect to wild-type cells, as loss of WRNIP1 does. Interestingly, comparing the percentage of restarting forks in all cell lines, we observed that the concomitant depletion of WRN and WRNIP1 reduces the ability of cells to resume replication after release from HU in the same extent as loss of WRNIP1 alone (Fig A). Thus, these results suggest that WRN and WRNIP1 may collaborate in a common pathway to restart stalled forks.

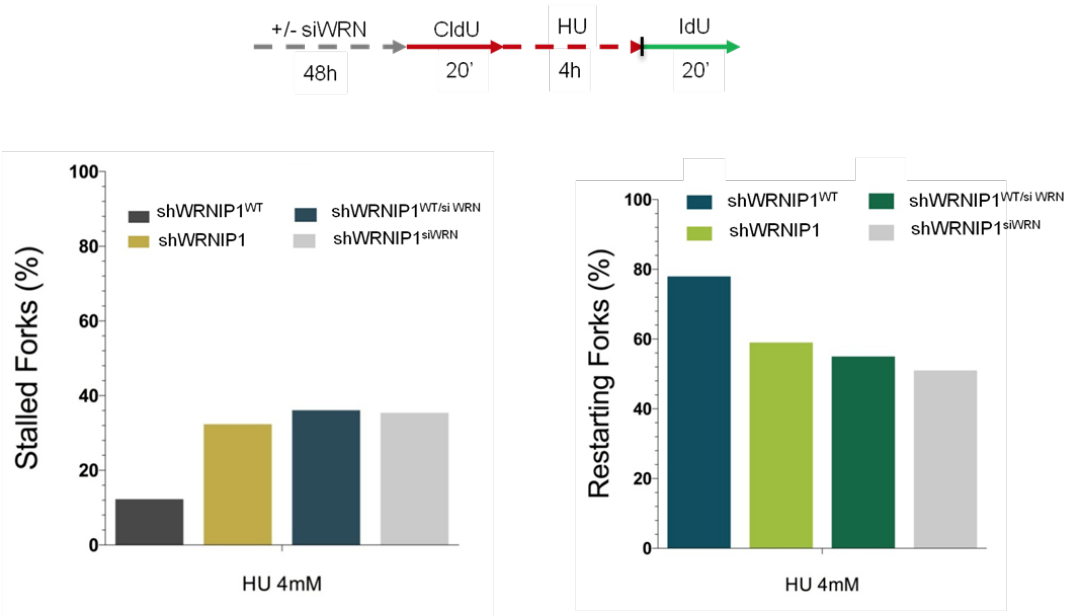

Figure A

However, in the new version of our discussion we have revised our conclusion, and we have tried to explain better why the WRNIP1/WRN/Polδ complex, whose association at stalled forks has been already proposed (Tsurimoto et al., Genes to Cells (2005) 10, 13-22), may be involved in replication fork restart, also taking into account previous studies showing that the complex can bind to stalled forks (Tsurimoto et al., Genes to Cells (2005) 10, 13-22), and can stimulate the DNA synthesizing activity of Polδ (Kamath-Loeb et al, PNAS (2000) 97, 4603-4608).

Accordingly, Polδ and WRN have been now removed from the cartoon showing the proposed model.

7) *The manuscript strictly needs revision to improve English phrasing and to include all relevant details (e.g. compounds, concentrations, timing of addition, etc.) in order to possibly reproduce the results. Much crucial information is not even included in the Supplementary material.*

We have carefully revised the manuscript to eliminate typing errors and improve understanding of the text. Moreover, we have corrected the "Experimental procedures" and "Supplemental Information" to include additional details about materials and procedures to better explain how our experiments have been performed.

Minor points:
1) *Fig. 3D looks to me pretty much redundant to Fig. 2B*

We agree with the referee's suggestion, and now, in the revised version of the manuscript, the Fig. 2B has been removed from the text. Accordingly, the figures have been reorganized.

2) *I don't quite understand the conclusion stressed by the authors from the data in Fig. 4, i.e. " Therefore, RAD51 is required for avoiding nascent strand degradation under stressful conditions in the absence of WRNIP1". I believe a far more relevant conclusion from the same data, which stresses the "physiological" role of the protein, would be that "WRNIP protects stalled forks by effective loading or retention of RAD51".*

We agree with the referee's suggestion, and in the revised version of the manuscript we amended the conclusion of the data set accordingly.

3) *I could not find among the references the Higgs et al., 2015 paper cited at page 16.*
We have carefully checked the list of references.

Answer to Referee #3's comments:
A large body of evidence indicates that stalled replication forks are processed by exonucleases such as Mre11 and that recombination factors like BRCA2 and Rad51 play a key role in preventing excessive resection of newly-replicated DNA. This control of Mre11-mediated resection at arrested forks is critical to prevent fork collapse and genomic instability. In this manuscript, Leuzzi and colleagues report the important observation that WRNIP1, a poorly-characterized interactor of the WRN helicase, acts together with BRCA2 and Rad51 to regulate Mre11 activity at HU-arrested forks. Using a combination of single-molecule and immunofluorescence-based assays, they report that WRNIP has two important functions at arrested forks. It prevents the displacement of Rad51 from arrested forks by the Fbh1 helicase in order to protect nascent DNA from nucleolytic degradation by Mre11. Moreover, it promotes the restart of stalled forks, presumably by interacting with Pol delta. Interestingly, the fork restart function of WRNIP1 depends on its ATPase activity, unlike its fork protection function. Finally, the authors show that the fork protection function of WRNIP1 is important to prevent replication-associated DNA damage and chromosome breaks. Overall, the data are of high quality and are presented in a clear and logical manner. Different assays are used in a clever manner to bring new insights into the mechanism of WRNIP1 action at stalled forks. Considering the growing interest on these new aspects of fork processing and restart, this study should be of a wide general interest to the readers of EMBO Journal. Yet, several important issues need to be addressed prior to publication.

We thank the referee for her/his useful criticisms to our work and for her/his appreciation of the manuscript. We have revised the manuscript taking into account the referee's suggestions (*see below*).

Specific issues:
1) *The experiments presented in this manuscript are generally well designed. However, a few important controls need to be shown and missing information should be provided. These include the percentage of transfected cells with WRNIP1 constructs and the effect of WRNIP1 depletion/complementation on the fraction of cells in S phase. Moreover, the authors should show how the original MRC5 cells compare to MRC5 cells expressing the shRNA against WRNIP1 and complemented with the wt protein, in terms of fork progression and arrest in the presence or the absence of HU.*

All cell lines used throughout the manuscript are stably transfected cell lines and are from single clones. We have now explained better in the "Experimental procedures" section how they have been generated.

To address the referee's concerns, we have provided a FACS analysis (Appendix Figure S12), in which we compared the effect of WRNIP1 depletion/complementation on the fraction of cells in S phase in shWRNIP1, shWRNIP1WT and shWRNIP1T294A cell lines, treated or not with HU. This analysis confirmed that there are no overt differences in cell cycle distribution with or without HU treatment in all cell lines.

2) *The DNA fiber experiments are convincing, but the authors should have used an antibody against ssDNA to label DNA fibers and show that terminally-arrested forks (e.g. in Fig 1C) do not correspond to broken fibers. In principle, this should be less of a problem for DNA fiber spreading than for DNA combing, but this control need to be performed anyway. Moreover, the concentration*

of IdU and CldU has to be indicated somewhere. The authors also need to make sure that the images shown are representative of the experiments. For instance, the CldU tracks show for the untreated control in Fig1E are 2 to 3 times shorter than IdU tracks, unlike what is shown below for the track length distribution.

For what concerns the DNA fiber assay, to check the quality of our samples, we are used to stain DNA with YOYO-1 green fluorescent dye before IF. As shown in the Fig. B-a, the staining with YOYO-1, performed soon after the spreading of the DNA fiber on the slides, suggests that we are able to get good quality DNA fibers, which are not damaged or broken. Indeed, as the referee says, the breakage of the DNA may be a more serious problem in the DNA combing technique rather than in DNA fiber assay. In addition, and unfortunately, the staining of DNA with YOYO-1 as well as with the anti-ssDNA antibody do not work well after immunofluorescence assay against CldU and IdU, so that it is recommended to check the DNA fibers initially as we and many labs do. However, as shown in Fig. B-b, our DNA fibers appear of good quality also after immunofluorescence staining with anti-CldU and anti-IdU antibodies, so that it allows us to be confident that adventitious fiber breakage does not undermine our replication dynamics analysis.

Nevertheless, according to the referee's recommendation, we provided images (Fig. B-c) showing that the antibody against ssDNA marks a DNA fiber (blue fluorescence), in which intact green fluorescence indicates the IdU labeling.

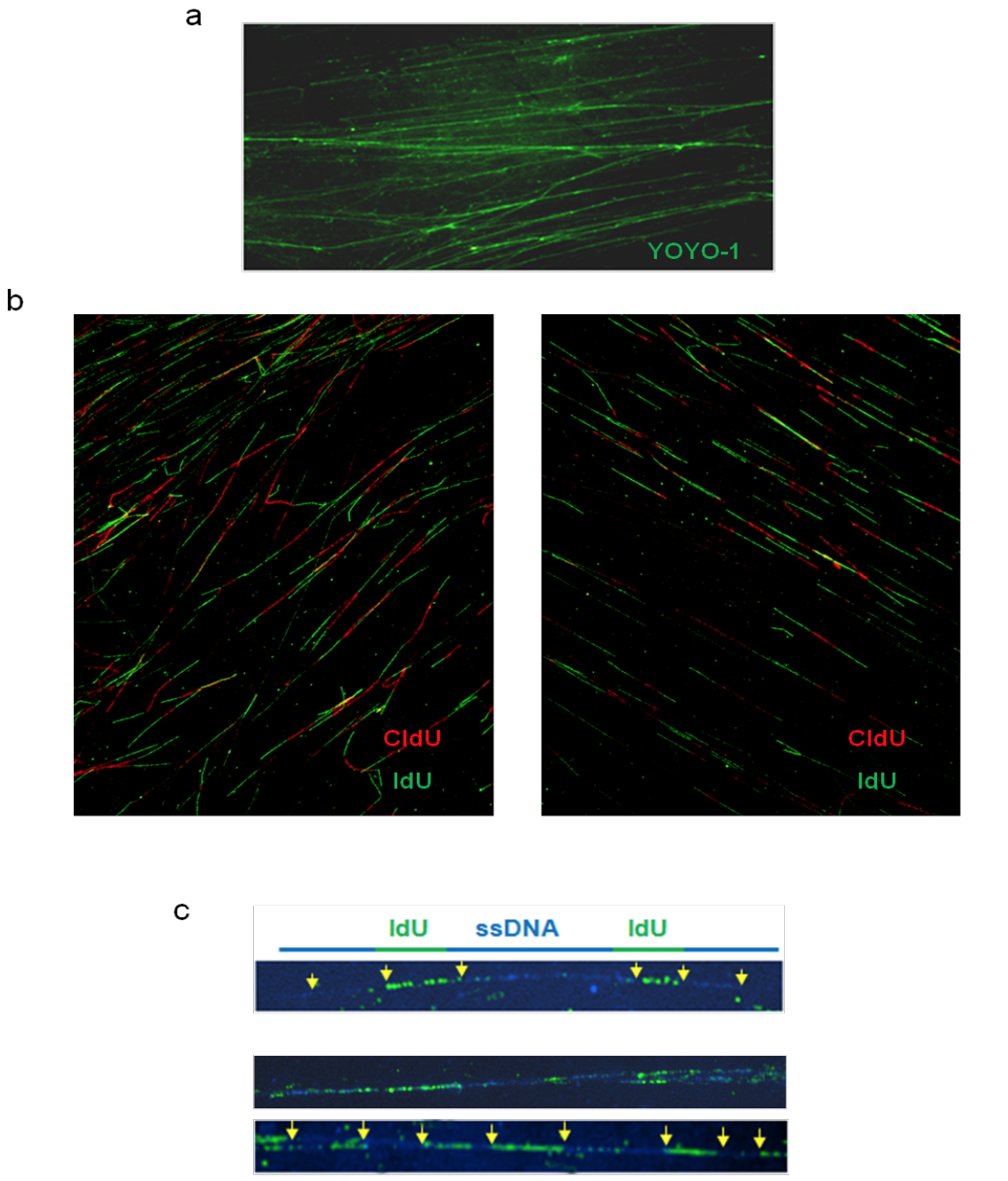

Figure B

For what concerns the protocol of the DNA fiber assay, we referred to a paper previously published by our group (Basile et al., NAR 2014). However, in the revised version of the manuscript, for the sake of clarity and for a better understanding of the experiments, we include more details in the "Experimental procedures" section about the protocols used. We have checked the images of DNA fibers and replaced those of Fig. 1H (Fig. 1E in the prior version of the manuscript) with more representative ones, according to the referee's suggestion.

3) *Fig 1C shows the percentage of stalled and restarting forks in WRNIP1-deficient and complemented cells. In principle, the sum of stalled and restarting forks should equal 1 for each category, but it does not seem to be the case. What do missing forks correspond to? Moreover, the expected patterns (red vs red-green) should be drawn in the same order as for the pulses for clarity (red-green and not gree-red).*

According to the referee's comment, we now provide graphs for new origins, terminating and interspersed forks, which were analyzed from the original version but not included in the corresponding graphs, thus explaining why the sum was not equal to 1 (Appendix Figure S1). Moreover, we have amended the experimental scheme of the Fig. 1E (Fig. 1C in the previous version of the manuscript).

4) *The supplementary tables showing statistical information for each DNA fiber experiment are nice, but they should indicate the number of the corresponding figure for each dataset. Numbers are missing at the end of table S1.*

In the revised version of the manuscript, we have amended the Table 1S according to the referee's critique.

5) *Figure 1F should be moved to sup data as it correspond to the only experiment performed with a different cell type.*

We agree with the referee's suggestion, and in the revised version of the manuscript the Fig 1F have moved in the "Supplementary data" section.

6) *Figure 3 shows that HU induces a significant increase of ssDNA production in cells complemented with the WT protein, but does not lead to the shortening of IdU tracks and to fork slow down. This apparent discrepancy should be discussed by the authors.*

Our immunofluorescence analysis shows that HU treatment induces an accumulation of ssDNA in wild-type cells in the absence of detectable fork degradation (Fig. 3 A). This is not at odds with our model. Indeed, a limited accumulation of ssDNA after HU in wild-type cells has been reported also by other investigators and can be explained by the uncoupling of MCM helicase and DNA polymerases, resulting in the exposure of ssDNA. Supporting this possibility, ssDNA is not linked to the MRE11-mediated fork degradation in wild-type cells. 13

7) *Figure 3F shows an HU-dependent accumulation of RAD51 on chromatin when WRNIP1 is present. However, there is a marked difference in RAD51 levels +/- HU in these cells, unlike in shWRNIP1 cells. This difference may suggest that WRNIP1 controls the synthesis/stability of RAD51, rather than its chromatin binding. The authors should use complementary approaches, such as IF-based assays, to strengthen their point. From this regard, the PLA assay is not entirely convincing, as it depends on the presence of ssDNA, whose formation at stalled forks is prevented by RAD51. This looks to me like a circular argument.*

We now confirmed the results obtained from the chromatin fractionation analysis of RAD51 by performing immunofluorescence analysis of RAD51 focal relocalization in shWRNIP1WT and shWRNIP1 cells treated or not with HU (Appendix Fig. S8).

Concerning the PLA assay reported in Fig. 3C (Fig. 3F in the previous version of the manuscript), we have now better explained that the decreased co-localization between ssDNA (anti-IdU signal) and RAD51 in WRNIP1-deficient cells after replication stress is the result of reduced levels of RAD51 in chromatin. In fact, the appearance of a PLA signal requires that both partners have to be present and close each other. Thus, even if more ssDNA is formed in shWRNIP1 cells the concomitantly reduced level of RAD51 precludes formation of PLA signal (Fig 3A).

8) *Important conclusions regarding the role of Mre11 and Rad51 are based on the use of inhibitors. Considering the specificity issues associated to the use of these inhibitors, these experiments should be confirmed with siRNAs.*

To further support our conclusions, we have confirmed experiments performed using chemical inhibitors by RNAi. The additional data are reported in Appendix Fig. S9 DNA fiber assay with siRAD51; and Appendix Fig. S5, and Appendix Fig. S14, for ssDNA experiments and chromosomal aberration analysis with siMRE11, respectively.

9) *Fig5B: is the difference between numbers of PLA spots +/- HU significant?*

In the revised version of the manuscript, we have included a statistical analysis for a better evaluation of this result.

10) *Fig6A: since gamma-H2AX foci form in S phase, the authors should provide FACS profiles to confirm that the differences they see are not due to differences in cell cycle distribution.*

According to the referee's suggestion, a monoparametric FACS analysis showing cell cycle distribution in shWRNIP1, shWRNIP1WT and shWRNIP1T294A cell lines, treated or not with HU, has been included (Appendix Fig S12).

11) *The authors repeatedly refer to the use of an anti-IdU antibody. However, all antibodies used to detect IdU and CldU were actually raised against BrdU, but show a different affinity for different halogenated nucleotides. This should be indicated in the methods section.*

We agree with the referee's suggestion, and the revised version of the manuscript was amended accordingly.

2nd Editorial Decision 30 March 2016

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see from the comments below, the referees both find that all major criticisms have been sufficiently addressed and recommend your manuscript for publication. However, they do have some remaining reservations with the conclusiveness of the data presented in the new figure 3D and ask you to discuss this point further. In addition, referee #2 suggests that you include any additional data that you may have at hand.

Given this shared concern from both refs, I would ask you to submit a final revision of your manuscript in which you discuss the data in fig 3D further. I would also encourage you to include more data - if already available - that could strengthen the reproducibility and statistical significance of the findings (although this will not be an absolute requirement from our side).

REFEREE REPORTS

Referee #2:

Regarding point 1), iPOND experiments would have made their points much stronger. I don't find the results in Figure 3D particularly convincing, even because it is not clear how many times the authors could reproduce those results and whether the differences are statistically significant.

Regarding point 6), I actually still think that whether WRNIP has a direct role in stabilizing the binding of RAD51 to ssDNA should have been tested in specific biochemical experiments, under controlled experimental conditions, in order to assess the added vaoue of WRMIP addition to the reaction. Also this important point is solely addressed by the results in Figure 3D, which do not look particularly convincing.

Should the authors in the meanwhile have obtained additional data to address the points above, the Editor may want to consider it (independently) as a valuable addition to the revised manuscript.

That said, the authors have certainly made a very reasonable job trying to address my main concerns and overall my impression is that this paper has reached the level of completion and solidity to be accepted for publication in the EMBO Journal.

Referee #3:

The authors have done a great work revising their manuscript and have properly addressed most of the issues raised by the referees. In my opinion, this ms is of wide general interest and should be published in EMBO Journal. Yet, an important issue needs to be addressed prior to publication. Referee #2 suggested to use a biochemical approach to address the recruitment of Mre11, Rad51 and WRNIP1 in the presence or shWRNIP1. To this end, the authors used an assay called CldU-co-IP

previously developed by others. The results shown in the new Fig.3D are convincing but the differences observed are not very strong and it is not clear how many times the experiment was performed (no error bars). Moreover, unlike the well-established iPOND technique using EdU and click chemistry, the IP of CldU requires that nascent DNA is at least partly single-stranded to be recognized by the antibody. It is therefore not clear to me what fraction of the forks is actually immunoprecipitated under these conditions. The authors at least comment on these issues or remove this figure from the manuscript.

2nd Revision - authors' response 19 April 2016

Response to Editors

We would like to thank very much you and the referees for your appreciation of the work we did to address the several concerns raised by the referees, in particular those from referees #2 and #3.
As suggested by the referees, in the final revised version of our manuscript, we have discussed further the data presented in Fig. 3D. We have specified that the experiment was repeated two times, introduced statistical analysis and explained the internal controls used for the CldU co-IP, to make our results more robust and convincing. We have also tried to explain better how the results reported in our manuscript support a role for WRNIP1 in stabilizing the binding of RAD51 to stalled replication forks, so that, in our opinion, further experiments could result unnecessary.

Below you can find a point-by-point detailed answer to the referees' concerns.

Response to Referees

Referee #2:
Regarding point 1), iPOND experiments would have made their points much stronger. I don't find the results in Figure 3D particularly convincing, even because it is not clear how many times the authors could reproduce those results and whether the differences are statistically significant.
Undoubtedly, iPOND is a very powerful method to purify proteins associated to replication forks. However, it requires a large amount of starting material and we reasoned that, as our analysis was biased towards two or three known proteins, the CldU-IP would have been a reasonable alternative approach. By the way, CldU-IP has been successfully used in many studies that analysed the presence of several proteins at replication forks. Of note, a recent iPOND-based study evidenced the presence of WRNIP1 at perturbed forks, supporting our confidence in our CldU-IP data.

For what concerns reproducibility of our CldU-IP data, we apologize for having not included statistical analysis in the original figure. Taking into account the referee's concerns, in the legend of Figure 3 D, it is now specified that the CldU-IP experiment was repeated two times, and the statistical analysis of the data is now provided in the graph. Our data demonstrate that RAD51 and MRE11 are differently recruited to stalled replication forks in the absence of WRNIP1, and that the differences in the loading of these proteins are statistically significant. In our opinion, the information shown in Western blot analysis can be now better understood and appreciated by the reader, and the results can be considered more robust and convincing. Moreover, in the revised version of the manuscript, we have further discussed our results.

Regarding point 6), I actually still think that whether WRNIP has a direct role in stabilizing the binding of RAD51 to ssDNA should have been tested in specific biochemical experiments, under controlled experimental conditions, in order to assess the added value of WRNIP addition to the reaction. Also this important point is solely addressed by the results in Figure 3D, which do not look particularly convincing.
Should the authors in the meanwhile have obtained additional data to address the points above, the Editor may want to consider it (independently) as a valuable addition to the revised manuscript.

We agree with the referee's comment and suggestion. Indeed, a set of in vitro experiments aimed to investigate if loading of RAD51 at ssDNA in the context of synthetic substrates mimicking stalled forks was affected by WRNIP1 is clearly intriguing. However, we reasoned that it would have required essentially a whole sets of experiments to be carefully executed, and that it would have been beyond the scope of a single manuscript too. Indeed, our data suggest an ordered involvement

of different proteins (i.e. RAD51, WRNIP1, BRCA2, FBH1), which would be not adequately modeled by two or three additional in vitro experiments; at least at the best of our knowledge and after discussion with some biophysicists from our department. It is our opinion that several in vivo experiments throughout the entire manuscript do support a role for WRNIP1 in stabilizing the binding of RAD51 to DNA. Indeed, we demonstrate that WRNIP1 co-immunoprecipitates with the BRCA2/RAD51 complex under both unperturbed and stressful conditions, and that physically interacts with RAD51. Since WRNIP1 directly interacts with RAD51, loss of this interaction may interfere with efficient nucleation of RAD51 on ssDNA in WRNIP1-deficient cells, thus undermining nascent strand integrity. Moreover, although WRNIP1-deficient cells show increased accumulation of ssDNA, an excess of RAD51 loaded on chromatin is not detected. In addition, loss of WRNIP1 leads to both reduced recruitment at stalled forks and association between ssDNA and RAD51. Consistently, concomitant depletion of WRNIP1 and RAD51 does not alter the excessive degradation occurring at stalled forks. However, RAD51 over-expression compensates for the excessive fork destabilization in WRNIP1-defective cells. Interestingly, depleting FBH1, which is involved in the removal of RAD51 from chromatin, both the fork degradation and chromosome instability phenotypes of WRNIP1-deficient cells are reverted, likely due to restoring of RAD51 levels in chromatin. In contrast, downregulation of FBH1 in BRCA2-deficient cells does not rescue fork degradation. Given that BRCA2, which mediates RAD51 loading to chromatin, is recruited correctly in WRNIP1-deficient cells, these experiments further support a role for WRNIP1 in stabilizing or retaining RAD51 at stalled forks.

That said, the authors have certainly made a very reasonable job trying to address my main concerns and overall my impression is that this paper has reached the level of completion and solidity to be accepted for publication in the EMBO Journal.

We thank very much the referee for her/his appreciation of the efforts we made to address all the concerns raised about our manuscript.

Referee #3:
The authors have done a great work revising their manuscript and have properly addressed most of the issues raised by the referees. In my opinion, this ms is of wide general interest and should be published in EMBO Journal. Yet, an important issue needs to be addressed prior to publication. Referee #2 suggested to use a biochemical approach to address the recruitment of Mre11, Rad51 and WRNIP1 in the presence or shWRNIP1. To this end, the authors used an assay called CldU-co-IP previously developed by others. The results shown in the new Fig.3D are convincing but the differences observed are not very strong and it is not clear how many times the experiment was performed (no error bars). Moreover, unlike the well-established iPOND technique using EdU and click chemistry, the IP of CldU requires that nascent DNA is at least partly single-stranded to be recognized by the antibody. It is therefore not clear to me what fraction of the forks is actually immunoprecipitated under these conditions. The authors at least comment on these issues or remove this figure from the manuscript.

We thank very much the referee for her/his appreciation of the efforts we made to address all the concerns raised about our manuscript.

For what concerns the comments about the CldU co-IP data, in the revised version of the manuscript, we have now specified that we repeated the experiments two times, and our statistical analysis shows that the differences in the loading of RAD51 and MRE11 are statistically significant. Moreover, we have further discussed our results. In our opinion, the information shown in Western blot analysis can be now better understood and appreciated by the reader, and the results can be considered more robust and convincing.

3rd Editorial Decision 28 June 2016

Thank you for submitting your re-revised manuscript with additional clarifications. We are happy to accept it now for The EMBO Journal but will still need a few minor modifications, for which I am returning the manuscript to you once more:

- as already discussed, please upload modified versions of Figures 1, 2 and 4, in which panels with low-resolution micrographs of DNA fiber assays have been replaced with reassembled figures.

- for the CldU-IP experiments in Figure 3D, it is important to state that these experiments have been replicated twice but please note that no meaningful statistics can be derived for N<3. The bar diagrams in the lower half of Fig. 3D should therefore be replaced by graphs showing the individual data points and a line indicating the mean, and no statements about statistical significance should be included. Please also see the respective section on statistical analysis in our Author Guidelines: http://emboj.embopress.org/authorguide#datapresentationformat

Following these final modifications, we should hopefully be in the position to swiftly proceed with formal acceptance and publication of the study.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Annapaola Franchitto
Journal Submitted to: The Embo Journal
Manuscript Number: EMBOJ-2015-93265R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For each experimental approach, the sample sizes are already specified in the text. The sample sizes were determined based on the standard requirements of each technique used in our study, and are similar to those generally reported in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes. To minimize the effects of subjective bias in the treatments, replicate of each treatment is done for the experiments.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes. To minimize the effects of subjective bias when assessing results, analysis was performed blinded by two independent investigators.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We applied the tests that are well accepted in the field to assess normality, i.e. D'Agostino Pearson test. In general, data from our experiments followed a normal distribution. If not, we used non-parametric tests to assess significance between groups.
Is there an estimate of variation within each group of data?	Yes. Data are reported as mean +/- SEM.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	EXPERIMENTAL PROCEDURES (Main text) In situ PLA assay (pag. 20) The primary antibodies used were: mouse-monoclonal anti-FLAG (clone M2, Sigma-Aldrich, 1:1000), rabbit-polyclonal anti-WRNIP1 (#GTX24731, GeneTex, 1:1000), rabbit-polyclonal anti-RAD51 ((#sc-8349, Santa Cruz Biotechnology, 1:500) and anti-IdU (mouse-monoclonal anti-BrdU/IdU; clone b44, Becton Dickinson, 1:10). SUPPLEMENTAL INFORMATION Co-immunoprecipitation, cell fractionation and Western blot analysis (pag. 1) Rabbit-polyclonal anti-WRNIP1 (#NB110-61626, Novus Biologicals, 1:2000), mouse-monoclonal anti-FLAG (clone M2, Sigma-Aldrich, 1:1000), mouse-monoclonal anti-GAPDH (clone 6C5, Millipore, 1:5000), rabbit-polyclonal anti-RAD51 (#sc-8349, Santa Cruz Biotechnology, 1:500), rabbit-polyclonal anti-LAMIN B1 (#sc-6216, Abcam, 1:10000), rabbit-polyclonal anti-BRCA2 (#A303-434A-M, Bethyl, 1:1000), mouse-monoclonal anti-MRE11 (clone 12D7, Novus Biological, 1:2000) and mouse-monoclonal anti-FBH1 (clone 2353C1a, Abcam, 1:200). Immunofluorescence (pag. 3) Mouse-monoclonal anti-γ-H2AX (clone JBW301, Millipore, 1:1000), rabbit-polyclonal anti-BRCA2 (#A303-434A-M, Bethyl, 1:1000) or rabbit-polyclonal anti-RAD51 (#sc-8349, Santa Cruz Biotechnology, 1:500).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cells used in our study were routinely checked for mycoplasma infection.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA