EMBO reports

CDC7 kinase promotes MRE11 fork processing, modulating fork speed and chromosomal breakage

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DOI: 10.15252/embr.201948920

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Review Timeline:

Submission Date:	22nd Jul 19
Editorial Decision:	26th Aug 19
Revision Received:	10th Feb 20
Editorial Decision:	23rd Apr 20
Revision Received:	29th Apr 20
Editorial Decision:	11th May 20
Revision Received:	12th May 20
Accepted:	13th May 20

Editor: Esther Schnapp

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 26th Aug 2019

Dear Dr. Santocanale,

Thank you for the transfer of your manuscript to EMBO reports. We have now received the full set of referee reports on it.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also all point out that the role of CDC7 in replication elongation must be separated from its role in replication initiation. Referees 1 and 2 further request that a potential role for Mre11 downstream of CDC7 needs to be strengthened.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 29,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view (EV) figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

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- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO Reports

Referee #1:

In this paper the authors examine the function of CDC7 in controlling replication elongation and stress responses. The authors observed that CDC7 inhibition reduced replication stress signaling, reduced nascent strand degradation in BRCA2-deficient cells, and altered replication fork restart. The authors conclude that these effects are due to direct effects of CDC7 on replication elongation and specifically on the function of the MRE11 nuclease. While this is possible, the authors do not rule out alternative explanations for the observations. In particular, CDC7 has an essential function in replication initiation. Changes in initiation could indirectly cause many of the effects observed. Thus, it is imperative for the authors to clearly and convincingly separate the putative role of CDC7 in controlling elongation from its function in initiation. In the absence of a clear separation of function, the data do not adequately support the conclusions.

Specific comments:

1. Cdc7 inhibition will inhibit origin firing and decrease the number of active replication forks. Why couldn't the results in figure 1 be explained by a reduction in replication initiation? A decrease in fork numbers would explain the reduction in signaling observed in Figure 1 as well as the reduction in DNA DSBs observed in Figure 1d. Thus, the conclusions that "CDC7 inhibition alters fork processing..." and "CDC7 activity is required for the transition from a stalled to a collapsed replication fork" on the second page of the results section cannot be made by the data presented up to that point. To make such a conclusion the authors would need to separate the well-established function of CDC7 in replication initiation from the proposed functions in elongation. This same issue creates problems in interpreting all of the data throughout the manuscript. The only place I could find where the authors acknowledge this problem is on the second page of the discussion where they state that the doses of CDC7i used in this work would have "limited effect" on origin firing. The evidence they point to for this statement is one of their previous publications which did not directly measure either the numbers of replication forks or rates of initiation. I also don't know what "limited effect" means. Any reduction in origin firing would yield changes in the experiments presented.

2. The conclusion that the mechanism of CDC7 function at active forks may be via regulation of MRE11 is based on very preliminary and limited data. While I would not expect a full description of mechanism in this paper, the data looking at MRE11 needs to be improved. For example, the statement that "Treatment with the CDC7 inhibitor dramatically reduced the degree of colocalization" is not an accurate description of the data presented. The amount of co-localization in the HU condition is less than 0.2 (Pearson's correlation), which is quite low already. It then changes to a mean of -0.1 with a very large error bar. I would not describe these as "dramatically" different. An accurate description of this data is needed. A second measure of Mre11 localization to replication forks should also be made such as measurement of co-localization of Mre11 with incorporated BrdU or with RPA.

3. In the DNA fiber analysis in figure 3 when was XL413 removed? If it was not removed then perhaps the reduction in red+green is actually due to a reduction in new origin firing. Dormant origins are known to fire during replication stress. These could rescue DNA synthesis in the absence of the XL413 treatment.

4. I am confused by the way the data in figure 3d is presented. Why show the ratio of IdU to CldU if the conclusion is about elongation rates? A ratio cannot be used to make a conclusion about the differences in speed between samples especially since the CPT treatment is shown to be in both labeling periods. In fact, if the only effect of CPT treatment were to increase fork reversal and slow elongation, then then both the CldU and IdU lengths should be shorter in this experiment which is not what the authors observed.

5. Please include more information in the figure legends and results section about how some experiments were performed. The methodology says that "In general, a 30 minutes pre-treatment with kinases inhibitors was used..." Was this how figure 1 was completed?

6. If CDC7 is acting at stalled replication forks to control the action of MRE11 or other proteins, then it should be possible to see effects of CDC7 inhibitor addition after HU addition. No pretreatment should be required.

7. In several cases it was unclear to me if the authors are equating "replisome stability" with "replication fork stability". The first refers to the protein machinery and the second refers to the DNA structure. They are not equivalent.

Referee #2:

In their manuscript titled "CDC7 kinase directs MRE11 processing at replication forks thereby modulating fork speed and chromosomal breakage upon genotoxic stress" Rainey et al report a series of interesting, novel and potentially important observations suggesting that Cdc7 kinase, in addition to its role in controlling DNA replication initiation, regulates a multitude of events at stalled replication forks in human cells.
They showed that Cdc7 chemical or genetic inhibition suppresses DSB formation and phosphorylation of DNA-damage response markers in response to HU. Chemical inhibition of Cdc7 impedes restart of replication forks previously stalled in HU and also the active slowdown of replication rate seen in low doses of CPT. Furthermore, they clearly showed that Cdc7 chemical inhibition has no effect on fork reversal, but it inhibits the excessive degradation of unprotected reversed forks in cells deficient for BRCA2. They suggest that this new emerging role of Cdc7 in

stalled replication is mediated by Cdc7-induced MRE11 re-localization to stalled replication factories.
Overall, this paper makes an important contribution towards the field and raises new hypotheses of Cdc7 function in DNA replication in human cells.

However, there are some concerns.

Major comments

a) MRE11 involvement. The manuscript strongly states that MRE11 mediates Cdc7 role at the stalled replication fork. Based on the literature, MRE11 may seem like the obvious candidate. However, the evidence presented here regarding MRE11 as the targeted nuclease controlled by Cdc7 was not convincing and needs strengthening by more direct experiments. Moreover, the specific involvement of MRE11 in each of the Cdc7-regulated processes put forward by this manuscript (fork restart in HU and slowdown replication rate in CPT mainly) was not clearly shown.

b) Cdc7 function in DNA replication initiation. The well-established role of Cdc7 in origin firing regulation could serve as an alternative explanation for some of the results presented here. The authors could address through other origin firing inhibitors (like CDK inhibitors). I realise that to fully address this concern would involve new experiments. However, I think that it is an important issue that the authors must minimally address somehow and acknowledge throughout the manuscript.

c) Lack of connection between the described Cdc7 regulated processes. Although the findings in this manuscript are novel and interesting for the field, the way the manuscript is presented seems more like a collection of phenotypes of Cdc7 inhibition than a cohesive story about Cdc7 involvement in stressed forks. The manuscript would greatly benefit from a more substantial discussion of the possible links between the different processes and a more holistic view of Cdc7 role in stalled forks.

Minor comments
Specific comments of MRE11 experiments in this manuscript:
In Figure 2
Panel B) MRE11 and EXO1 chromatin levels shown here seems to be constant regardless long HU treatments and ATM inhibition.

Panel C) The MRE11 foci depicted here, are S phase dependent? Are HU dependent? Are there some difference in the amount of foci in those conditions?

Such data would greatly strengthen this paper and the make the findings and conclusions involving MRE11 more than speculative.

Referee #3:

This paper addresses the role of the CDC7 kinase in stabilising and restarting stalled replication forks. There has been discussion of a possible role for CDC7 in fork stability for many years, but firm evidence and clear mechanisms have been lacking. Of particular interest is the observation in this

paper that CDC7 acts epistatically with MRE11, both being involved in mechanisms that degrade nascent DNA at stalled or reversed forks. This role in processing of nascent DNA could partially explain CDC7's role in fork stabilisation. I think the work provides some important new evidence about the role of CDC7 which mighty also be of relevance for understanding how CDC7 inhibition might be used therapeutically. However, I have a major concern about the interpretation of the data in Figures 1 and 5 which would need to be addressed before publication.

One difficulty in studying a role for CDC7 in fork stability is the need to clearly distinguish this from CDC7's well-studied role in replication fork initiation. Because CDC7 is required for replication initiation, it is unsurprising that markers of DNA damage or replication stress are reduced when CDC7 is inhibited, because there are fewer forks to generating these marks. Although the authors are aware of this problem, I think Figures 1 and 5 are not adequately controlled to distinguish effects on fork number from fork stability.

The authors conclude from the data in Fig 1 that because CDC7 inhibition suppresses RPA2 and H2AX phosphorylation in response to HU, CDC7 alters processing of stalled forks. However, it is more likely that this effect is mediated by there being fewer forks to respond to HU when CDC7 is inhibited. This experiment needs to show that the effect of CDC7 inhibition on fork stability is separable from its effect on fork stabilisation, as for example has been done in Xenopus (Alver et al). It is surprising that this paper is not cited, particularly because the authors go on to say 'While there is currently no evidence of CDC7 affecting replisome stability...', which is one of the main conclusions of the Alver et al paper ('Reversal of DDK-Mediated MCM Phosphorylation by Rif1-PP1 Regulates Replication Initiation and Replisome Stability Independently of ATR/Chk1').

I have similar problems for Fig 5: cells were treated with HU plus or minus CDC7 inhibition for 5 hr, and then were released into mitosis and the number of chromosome breaks was measured. CDC7 inhibition reduced the number of HU-induced breaks, but this is most likely explained by the effect of CDC7 on inhibiting initiation so there are fewer forks active in the period when cells are exposed to HU. Note that the data in Figs 3 - 4 about the role of CDC7 in the processing of stalled forks do not suffer this objection as they are fibre assays investigating individual replicating strands. These experiments though are somewhat similar to the results recently reported by Sasi et al, though some of the details are different (for example the effect of HU on degradation).

Minor Point
Fig 2a: the EXO1 blot shows 2 bands: which is EXO1, and what is the evidence for the specificity of the antibody?

Referee #1:

In this paper the authors examine the function of CDC7 in controlling replication elongation and stress responses. The authors observed that CDC7 inhibition reduced replication stress signaling, reduced nascent strand degradation in BRCA2-deficient cells, and altered replication fork restart. The authors conclude that these effects are due to direct effects of CDC7 on replication elongation and specifically on the function of the MRE11 nuclease. While this is possible, the authors do not rule out alternative explanations for the observations. In particular, CDC7 has an essential function in replication initiation. Changes in initiation could indirectly cause many of the effects observed. Thus, it is imperative for the authors to clearly and convincingly separate the putative role of CDC7 in controlling elongation from its function in initiation. In the absence of a clear separation of function, the data do not adequately support the conclusions.

Specific comments:

1. Cdc7 inhibition will inhibit origin firing and decrease the number of active replication forks. Why couldn't the results in figure 1 be explained by a reduction in replication initiation? A decrease in fork numbers would explain the reduction in signaling observed in Figure 1 as well as the reduction in DNA DSBs observed in Figure 1d. Thus, the conclusions that "CDC7 inhibition alters fork processing..." and "CDC7 activity is required for the transition from a stalled to a collapsed replication fork" on the second page of the results section cannot be made by the data presented up to that point. To make such a conclusion the authors would need to separate the well-established function of CDC7 in replication initiation from the proposed functions in elongation. This same issue creates problems in interpreting all of the data throughout the manuscript. The only place I could find where the authors acknowledge this problem is on the second page of the discussion where they state that the doses of CDC7i used in this work would have "limited effect" on origin firing. The evidence they point to for this statement is one of their previous publications which did not directly measure either the numbers of replication forks or rates of initiation. I also don't know what "limited effect" means. Any reduction in origin firing would yield changes in the experiments presented.

We have thoroughly revised the manuscript acknowledging the possibility that the reduction in origin firing could, at least partially, account for the reduction of DSB and RPA phosphorylation observed in figure 1. However, we have also directly tackled this issue, including a substantial set of new experiments which strongly indicate that CDC7 function at forks can be separated from origin firing. These can be recapitulated as follows:
a) Using cells that have been arrested in HU in the presence of CDC7i, we show that upon removal of CDC7i H2AX phosphorylation is induced in absence of further origin firing, which was monitored by both CDC45 loading to chromatin and EdU incorporation. We further demonstrated that the H2AX phosphorylation observed upon removal of the CDC7i is mostly dependent on MRE11 activity (new Fig 2).
b) DNA damage in HU can be efficiently suppressed by low doses of CDC7 inhibitor, which have barely detectable effects on DNA synthesis (new Fig EV2) without HU , indicating a

different level of requirement for CDC7 activity in promoting efficient DNA synthesis and in driving HU dependent H2AX phosphorylation.

Besides uncoupling effects at origins and forks, these data suggest that key targets of CDC7 activity in initiation and elongation may be differently regulated.

Finally, as pointed out by Rev#3, all experiments in Figs 4-5 monitor the fate of previously established forks, and should thus not be affected by CDC7 role in initiation.

2. The conclusion that the mechanism of CDC7 function at active forks may be via regulation of MRE11 is based on very preliminary and limited data. While I would not expect a full description of mechanism in this paper, the data looking at MRE11 needs to be improved. For example, the statement that "Treatment with the CDC7 inhibitor dramatically reduced the degree of colocalization" is not an accurate description of the data presented. The amount of co-localization in the HU condition is less than 0.2 (Pearson's correlation), which is quite low already. It then changes to a mean of -0.1 with a very large error bar. I would not describe these as "dramatically" different. An accurate description of this data is needed. A second measure of Mre11 localization to replication forks should also be made such as measurement of co-localization of Mre11 with incorporated BrdU or with RPA.

We have now addressed the role of CDC7 in the recruitment and maintenance of MRE11 at forks and replication factories. We have performed several Dm-ChP (iPond) experiments which show that both proteins are at forks and retained in HU. While this was known for MRE11 this is the first time that it is directly shown for CDC7 (new Fig 3A-C).
Importantly we find that both MRE11 and CDC7 retention/accumulation at forks is partially impaired in the presence of CDC7i, which correlates with a differential phosphorylation dependent mobility shift of the MRE11 protein (new Fig 3D).

These new biochemical data are consistent with our IF data on co-localization between MRE11 and with PCNA, which we have further expanded with a new set of experiments and increasing sample size, thereby reducing error bars.

We have performed colocalization of MRE11 with RPA2 (new Fig EV3). This is also reduced by CDC7 inhibition in HU albeit to a lesser extent that MRE11/PCNA colocalization.

New functional experiments, now shown in Fig 2 (discussed above) and in Fig 4B and D, further strengthen the idea that CDC7 effects of forks are mediated by MRE11.

3. In the DNA fiber analysis in figure 3 when was XL413 removed? If it was not removed then perhaps the reduction in red+green is actually due to a reduction in new origin firing. Dormant origins are known to fire during replication stress. These could rescue DNA synthesis in the absence of the XL413 treatment.

In the fork restart experiments (previously in figure 3 and now in Fig 4A-B), XL413 was present throughout. When the medium containing HU and XL413 was removed, it was replaced with fresh medium which contained XL413 and CldU.

4. I am confused by the way the data in figure 3d is presented. Why show the ratio of IdU to CldU if the conclusion is about elongation rates? A ratio cannot be used to make a conclusion about the differences in speed between samples especially since the CPT treatment is shown to be in both labeling periods. In fact, if the only effect of CPT treatment were to increase fork reversal and slow elongation, then then both the CldU and IdU lengths should be shorter in this experiment which is not what the authors observed.

Please note that in these experiments CPT was added during the second labelling (with IdU) thus only IdU labelled tracks are expected to be shorter, while CldU track length is unaffected by drug treatment and acts as internal control. In that respect, ratios of IdU/CldU track lengths is thus a simple, well-established readout to assess active fork slowing by mild genotoxic treatments. We have rephased the text and figure legend for clarity.

5. Please include more information in the figure legends and results section about how some experiments were performed. The methodology says that "In general, a 30 minutes pre-treatment with kinases inhibitors was used..." Was this how figure 1 was completed?

We have revised figure legends and M&M section to provide clear information on the methodology.

6. If CDC7 is acting at stalled replication forks to control the action of MRE11 or other proteins, then it should be possible to see effects of CDC7 inhibitor addition after HU addition. No pretreatment should be required.

This is has proven an interesting and important point for the manuscript and its interpretation.
We have included an ad-hoc experiment in new Fig 1D which shows that the pre-treatment is important to prevent DNA damage in HU. While this could simply do to pharmacological reasons in the kinetics of compound uptake by the cells, more likely it points out to the fact that the CDC7 dependent phosphorylation has firstly to be removed by a phosphatase, in order to reduce fork processing and its destabilization.
These data are now reinforced in the experiments where we show that reactivation of CDC7 in HU treated cells can cause DNA damage that is mostly dependent on MRE11 (Fig 2)

7. In several cases it was unclear to me if the authors are equating "replisome stability" with "replication fork stability". The first refers to the protein machinery and the second refers to the DNA structure. They are not equivalent.

We have revised the manuscript accordingly.

Referee #2:

In their manuscript titled "CDC7 kinase directs MRE11 processing at replication forks thereby modulating fork speed and chromosomal breakage upon genotoxic stress" Rainey et al report a series of interesting, novel and potentially important observations suggesting that Cdc7 kinase, in addition to its role in controlling DNA replication initiation, regulates a multitude of events at stalled replication forks in human cells.
They showed that Cdc7 chemical or genetic inhibition suppresses DSB formation and phosphorylation of DNA-damage response markers in response to HU. Chemical inhibition of Cdc7 impedes restart of replication forks previously stalled in HU and also the active slowdown of replication rate seen in low doses of CPT. Furthermore, they clearly showed that Cdc7 chemical inhibition has no effect on fork reversal, but it inhibits the excessive degradation of unprotected reversed forks in cells deficient for BRCA2. They suggest that this new emerging role of Cdc7 in stalled replication is mediated by Cdc7-induced MRE11 re-localization to stalled replication factories.
Overall, this paper makes an important contribution towards the field and raises new hypotheses of Cdc7 function in DNA replication in human cells.

However, there are some concerns.

Major comments

a) MRE11 involvement. The manuscript strongly states that MRE11 mediates Cdc7 role at the stalled replication fork. Based on the literature, MRE11 may seem like the obvious candidate. However, the evidence presented here regarding MRE11 as the targeted nuclease controlled by Cdc7 was not convincing and needs strengthening by more direct experiments. Moreover, the specific involvement of MRE11 in each of the Cdc7-regulated processes put forward by this manuscript (fork restart in HU and slowdown replication rate in CPT mainly) was not clearly shown.

We have reinforced the connection between CDC7 and the MRE11 dependent processes at forks in multiple sections and figures in the manuscript.
Specifically:

1) in Fig 2 we show that the DNA damage (γH2AX) at established forks in HU , which is prevented by CDC7i, is also mostly prevented by MRE11 inhibition by Mirin.
2) We have strengthened the finding that fork slowdown in CPT is MRE11-dependent by adding a new set of experiments in which MRE11 is downregulated by siRNA, which further confirm the previous findings obtained with Mirin. In this assay CDC7 inhibition phenocopies MRE11 inhibition and depletion (new Fig 4C-D).
3) MRE11 involvement in fork restart was also previously shown in (Bryant et al. 2009- EMBO J. 28: 2601–15). We now confirm this data showing that fork restart from HU is defective if MRE11 is inhibited by Mirin and in a different cell line (U2OS) (Fig 4B).

b) Cdc7 function in DNA replication initiation. The well-established role of Cdc7 in origin firing regulation could serve as an alternative explanation for some of the results presented here. The authors could address through other origin firing inhibitors (like CDK inhibitors). I

realise that to fully address this concern would involve new experiments. However, I think that it is an important issue that the authors must minimally address somehow and acknowledge throughout the manuscript.

We have thoroughly addressed this point with the experiments now reported in Fig 2, Fig EV2 and text. We kindly refer this reviewer to our response to Rev #1, who had a similar comment (point 1).

c) Lack of connection between the described Cdc7 regulated processes. Although the findings in this manuscript are novel and interesting for the field, the way the manuscript is presented seems more like a collection of phenotypes of Cdc7 inhibition than a cohesive story about Cdc7 involvement in stressed forks. The manuscript would greatly benefit from a more substantial discussion of the possible links between the different processes and a more holistic view of Cdc7 role in stalled forks.

We have revised the discussion in its last section taking in consideration this reviewer comment, and thus linking the phenotypes observed and suggesting a possible mechanisms by which these could be regulated by CDC7.

Minor comments
Specific comments of MRE11 experiments in this manuscript:
In Figure 2
Panel B) MRE11 and EXO1 chromatin levels shown here seems to be constant regardless long HU treatments and ATM inhibition.

This experiment has been removed, as more compelling experiments (Dm-ChP/iPond) which specifically assess protein occupancy at forks have now been included (Fig 3A-C)

Panel C) The MRE11 foci depicted here, are S phase dependent? Are HU dependent? Are there some difference in the amount of foci in those conditions?

MRE11 can be detected in nuclear foci in HU and throughout the cell cycle with a different pattern. This has been studied in detail in (Mirzoeva & Petrini Mol Cell Biol. 2001 Jan;21(1):281-8). We have not further extended these studies.

Such data would greatly strengthen this paper and the make the findings and conclusions involving MRE11 more than speculative.

We have further strengthened MRE11 involvement with the additional data in Fig 2 A-E, Fig 3A-D, Fig 4B and 4D.

Referee #3:

This paper addresses the role of the CDC7 kinase in stabilising and restarting stalled replication forks. There has been discussion of a possible role for CDC7 in fork stability for many years, but firm evidence and clear mechanisms have been lacking. Of particular interest is the observation in this paper that CDC7 acts epistatically with MRE11, both being involved in mechanisms that degrade nascent DNA at stalled or reversed forks. This role in processing of nascent DNA could partially explain CDC7's role in fork stabilisation. I think the work provides some important new evidence about the role of CDC7 which mighty also be of relevance for understanding how CDC7 inhibition might be used therapeutically. However, I have a major concern about the interpretation of the data in Figures 1 and 5 which would need to be addressed before publication.

One difficulty in studying a role for CDC7 in fork stability is the need to clearly distinguish this from CDC7's well-studied role in replication fork initiation. Because CDC7 is required for replication initiation, it is unsurprising that markers of DNA damage or replication stress are reduced when CDC7 is inhibited, because there are fewer forks to generating these marks. Although the authors are aware of this problem, I think Figures 1 and 5 are not adequately controlled to distinguish effects on fork number from fork stability.

We have thoroughly address this point with the experiments now reported in Fig 2 and fig EV2 and text. We kindly refer this reviewer to our response to Rev #1, who had a similar comment (point 1).

The authors conclude from the data in Fig 1 that because CDC7 inhibition suppresses RPA2 and H2AX phosphorylation in response to HU, CDC7 alters processing of stalled forks. However, it is more likely that this effect is mediated by there being fewer forks to respond to HU when CDC7 is inhibited. This experiment needs to show that the effect of CDC7 inhibition on fork stability is separable from its effect on fork stabilisation, as for example has been done in Xenopus (Alver et al). It is surprising that this paper is not cited, particularly because the authors go on to say 'While there is currently no evidence of CDC7 affecting replisome stability...', which is one of the main conclusions of the Alver et al paper ('Reversal of DDK-Mediated MCM Phosphorylation by Rif1-PP1 Regulates Replication Initiation and Replisome Stability Independently of ATR/Chk1').

We apologise for missing to report the findings of this paper in our first submission, which indeed are closely related to our work. This has been now properly cited in the introduction and further discussed in the discussion section.

I have similar problems for Fig 5: cells were treated with HU plus or minus CDC7 inhibition for 5 hr, and then were released into mitosis and the number of chromosome breaks was measured. CDC7 inhibition reduced the number of HU-induced breaks, but this is most likely explained by the effect of CDC7 on inhibiting initiation so there are fewer forks active in the period when cells are exposed to HU. Note that the data in Figs 3 - 4 about the role of CDC7 in the processing of stalled forks do not suffer this objection as they are fibre assays investigating individual replicating strands. These experiments though are somewhat similar

to the results recently reported by Sasi et al, though some of the details are different (for example the effect of HU on degradation).

We agree with this reviewer that the number of HU-induced chromosomal breaks can be affected by the number of fork present during the 5 h incubation with the drug. Importantly the number of chromosomal breaks occurring in BRCA2 depleted cells within a full round of replication without HU is also reduced by CDC7i (Fig 6D-E). As these breaks occur by defective processing of unprotected forks their number should be proportional to the number of events of fork stalling/processing and amount of DNA synthetized more than the number of forks generated. Compared to the first submission we have reinforced this data by showing the same phenotype in an additional cell line (Fig 6D) .

Minor Point
Fig 2a: the EXO1 blot shows 2 bands: which is EXO1, and what is the evidence for the specificity of the antibody?

Both bands disappear upon EXO1 depletion by siRNA although with a distinct kinetics (data included in Appendix Fig 2B). At this stage we can reasonably suggest that the two bands may represents two alternative EXO1 isoforms derived from alternative splicing (Uniport Q9UQ84 and Q9UQ84-4).

1st Revision - Editorial Decision 23rd Apr 2020

Dear Corrado,

Thank you for your patience while your revised manuscript was peer-reviewed. I asked referee 3 to please assess how well all referee concerns were addressed, and I am happy to say that s/he supports the publication of your study now. Only a few more minor changes will be required before we can proceed with the official acceptance of your study:

Please address referee 3's last concern.

Our in-house figure check concluded that all blots are overcontrasted, please send better pictures with your final manuscript.

Please also send us the original pictures (source data) of the gel bands shown in Fig 5B.

The EMBO reports reference style will change on the 1st of May. If you can send us the final manuscript before the 30th of April, it can keep the numbered reference style. If the manuscript will be submitted later, please change the reference style to the new one (there are links to both styles in our guide to authors online).

The APPENDIX table of content is missing page numbers, please add. The nomenclature needs correcting to 'Appendix Figure S#' and 'Appendix Table S#'. Please also correct the Appendix callouts in the manuscript text.
Appendix Fig S3 needs a scale bar.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #3:

The authors have added important new information to the paper that provides good evidence that the effect of CDC7 inhibition on DNA damage markers is independent of the role of CDC7 in promoting the initiation of replication. Figure EV2 shws that at a low dose of XL413 the reduction of

gamma-H2AX is much greater than the reduction of EdU incorporation; if the two were co-dependent, the two responses would be expected to be proportional. In Fig2, cells are treated with HU and XL413 to stabilise fork number, and then if the XL413 is removed, and an increase in gamma-H2AX is seen. Both of these experiments suggest that the decrease in gamma-H2AX caused by CDC7 inhibition is not mediated by a decrease in fork number mediated by CDC7's known role in replication initiation.

The experiments shown in Figure 6 still do not separate the potential functions of CDC7 in initiation and in fork stability, but given the additional data in Figures and EV2, I think the likelihood of this reflecting a fork stability function is much higher. However, I still think the authors should show some caution in the interpretation of this experiment: for example, the very last sentence of the Results could be amended to say: "Although it is possible that the effect of CDC7 inhibition on decreasing chromosome breaks might be in part mediated by a reduction in the number of active forks, these results are also consistent with the idea that, in absence of fork protection, CDC7 kinase promotes MRE11 nuclease attack of reversed forks leading to chromosomal instability."

With that single change, I think the paper is now suitable for publication.

Dear Esther,

I have resubmitted the manuscript that has been revised taking in account Reviewer 3 comment as well as your and data editor indications. All changes are tracked in the main text file.

As I mentioned in my previous email the blots performed in Galway are digital reproductions generated by the scanning and quantification of membranes using an Odyssey imaging and Image studio software. Compared to classical chemoluminescence detection on films, bands tend to be sharper. We have adjusted the levels with some improvements in some of these images- As an example I have uploaded several TIFF files related to the blots in Fig 1C which were directly exported from the Image studio software and representing different levels.

Fig 5B is the exception and that experiment was performed in Zurich with chemiluminescence and Fusion Solo imaging system. We have uploaded several Tiff files of the original images representing different exposures.

I hope that everything is fine and the manuscript is now acceptable for publication.

Best wishes,
Corrado

2nd Revision - Editorial Decision 11th May 2020

Dear Corrado,

As discussed, please modify the statistical analyses of the figures 1B, 4C, 4D, 5C, 5F and EV1C, and please explain in the figure legends what the statistics are based on.

I am making another "revise only" decision, so that you can upload the new figures.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

12/05/2020

Dear Esther,

I have just uploaded the new file and resubmitted the manuscript. We have made the changes we previously discussed and uploaded the source data for every figure as separate PDF files. All changes are summarised below and tracked in the text.

Figure 1: Statistics removed from graph in Figure 1B

Figure 2: No change

Figure 3: panel 3D – bottom (lambda phosphatase experiment)- was updated, a lane removed from original figure. This is due to a miscommunication on how the experiment was originally preformed. There is no change in the significance of the experiment. Text and figure legend updated accordingly.

Figure 4: we clarified in figure legend that the statistics is related to the experiment shown and that similar results were obtained in a second independent experiment.

Figure 5: We removed the statistics from panel 5C and included a new graph (5D) with the stats of three different experiments.
We removed stats from Fig 5G (former 5F).

Figure 6: No change

Figure EV1: Statistics removed from graph in Figure EV1C

Figure EV2: No change

Figure EV3: No change

Figure Appendix S1: No change

Figure Appendix S2: No change

Figure Appendix S3: No change

Appendix Table S1: No change

I hope that this work is now suitable for publication on EMBO reports and I would like to thank you again for handling the manuscript.

Looking forward to hearing from you.

Corrado Santocanale

3rd Revision - Editorial Decision 13th May 2020

Prof. Corrado Santocanale
National University of Ireland Galway
Centre for Chromosome Biology
NUIG
University Road
Galway, Galway 000
Ireland

Dear Prof. Santocanale,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Corrado Santocanale
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48920V2

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 iustified
- ➔ 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 χ2 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	Based on previous studies and all statistics analysed with GraphPad PRISM
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.	NA
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.	NO
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.	Yes
Is there an estimate of variation within each group of data?	Yes

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http://www.ncbi.nlm.nih.gov/gap

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	All statistics analysed with GraphPad PRISM

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).	YES
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U20S and MCF10A celles were purchased from ATCC, AS-CDC7 were generated in the Santocanale lab and previoulsy described. All cells lines were tested for mycoplasma contamination every 6 months

* 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
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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 a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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).	Upon acceptance
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G- Dual use research of concern

22. 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