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Chk1 loss creates replication barriers that compromise cell survival independently of excess origin firing

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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 17th January 2019

Thank you for submitting your manuscript for our editorial consideration, and please excuse our delayed response due to the high number of submissions and limited referee availability before/during the holiday period. We have now received reports from three expert reviewers, copied below for your information. As you will see, the referees consider your study and its results interesting in principle, but they also raise a substantial number of major concerns that would need to be decisively clarified before the paper may become suitable for publication. In particular, it would seem essential to test and rule out various alternative scenarios/explanations for the observed findings, such as replication-transcription conflicts (ref 1) or MUS81-EME1-mediated effects (ref 3). Two other key points are the incompletely established role and regulation of Pol eta in this context (refs 1 & 2), and the unclear role of CDC45 (refs 1& 3). In addition, all referees retain reservations regarding the general narrative, and the presentation and statistical analysis of the data. In light of these significant issues, I am afraid that the study does at this stage not yet appear as a sufficiently strong candidate for an EMBO Journal article.

Given the overall interest expressed by the referees and the fact that all of them offer constructive suggestions for deepening the insights and improving the conclusiveness of this work, I would nevertheless like to give you an opportunity to respond to the reviewers' comments by way of a revised version of the manuscript. I should however point out that given that it is not clear whether all key criticisms (as well as the various more specific points) may be adequately addressable during the single round of major revision we normally allow for, and since the required revision experiments may also confound, rather than corroborate, the original conclusions, I am not in a position to make strong predictions on the outcome of eventual reevaluation by our referees in this case. In any case, I would be happy to discuss a possible extension of our normal three-months 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. Also, should you have any specific questions/comments regarding the referee reports or the revision requirements, please do not hesitate

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to get in touch with me already during the early stages of your revision work.

REFEREE REPORTS

Referee #1:

In this manuscript, Gonzalez Besteiro et al analyze the dynamics of DNA replication following the downregulation of effector checkpoint kinase Chk1, building upon the established notion that Chk1 inhibition promotes extra origin activation and a global reduction in fork speed.

The authors use an interesting variation of the standard assay that monitors DNA replication in individual DNA molecules to show that the effect on fork progression in Chk1-deficient cells is not due to reduced fork speed but to a higher frequency of fork stalling events (Figures 1-2). They report PCNA monoubiquitination and the presence of TLS polymerase eta (Pol eta) at forks (Figure S1G; the iPOND experiment, however, indicates that Pol eta is present at forks regardless of Chk1 downregulation). As loss of Chk1 is known to unleash CDK2 activity, roscovitin is added to shChk1-treated cells to partially rescue fork stalling and excess origin firing. Fork progression is also partially rescued by the addition of extra nucleosides, and this effect depends on Pol eta (Figures 3 and S2). Expression of GFP-tagged Pol eta also rescues fork progression to some extent, whereas a phosphomimetic mutant in Ser687 does not, suggesting an inhibitory role for this phosphorylated residue (Figure 4 and S3). The effects of Chk1 on fork rate and origin activation can be separated and are not interdependent, contrary to other reports (Figures 5 and S4). Rescue of fork progression with nucleosides or exogenous Pol eta does not eliminate the DNA damage caused by Chk1 inhibition, whereas reducing the number of active origins by targeting CDC7 or CDT1 does (Figures 6, S5 and S6). Finally, the authors show that targeting CDC45 expression (a component of the CMG helicase) is sufficient to restore normal fork rate, origin firing and cell survival upon loss of Chk1 (Figure 7 and S7).

The topic is interesting and the experiments are generally well designed and executed. The study provides several insights into the DNA replication defects caused by the loss of Chk1 activity. I found it difficult, however, to integrate them into a clear central message. I think the article needs to be revised before being considered for publication.

Main comments:

1. There is no conclusive information about the role of Pol eta in the context of Chk1 loss: an inhibitory phosphorylation at Ser687 may prevent its function at bypassing replication barriers (Fig 4), but the relevance of this mechanism is unclear as fork progression can be rescued by Pol eta expression without eliminating DNA damage or cell death (Fig S5). As acknowledged in the Discussion, the phosphorylation at Ser687 may play different functions, e.g. promote or repress the bypass of lesions or barriers, depending on the specific cellular context.

2. All the data suggest that the main phenotypes caused by loss of Chk1 are derived from an excess in origin firing, which in turn creates "replication barriers" of unknown nature. Can the authors rule out that these barriers are actually conflicts with the transcription machinery? This seems to be the mechanism underlying fork collapse when excess origins are activated by oncogenic stimuli (Macheret and Halazonetis, Nature 2018). The authors may be able to test whether the number of R-loops is elevated, or whether the phenotype of Chk1 loss can be alleviated by an inhibitor of transcription elongation.

3. It is clear that downregulation of Cdc45 partially rescues the phenotypes caused by Chk1 inactivation because it reduces the percentage of active origins. However, the concept that "replication barriers are created by excess CDC45" (Discussion, first paragraph) is an overstatement. No evidence is provided to support that an excess of CDC45 creates non-B DNA structures. Even the notion that CDC45 is bound to DNA in excess following Chk1 downregulation relies on observations from other groups and should be independently confirmed in the authors' cellular

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system. The experiment shown in Figure 7E shows total levels of CDC45, not chromatin-bound levels, and siLuc and siChk1 are shown in different panels.

Other minor points:

1. p3 (Introduction), the sentence "In unperturbed conditions, Chk1 loss triggers excess origin firing and reduced rates of fork elongation" should also cite earlier work from the Lukas-Bartek lab (Syljuasen et al MCB 2005).

2. p8, l4, "pulses of IdU (10-100m)" should read "(10-80m)"

3. p15, l7 from bottom, the presence of ssDNA is referred to as a marker of "replication catastrophe". This term might have been used in other papers, but it seems more reasonable to use "replication stress".

4. p20, the sentence "The DNA fiber assay is insensitive to such localized sites..." is not clear.

5. Reference Rodriguez-Acebes et al (JBC 2018) is incomplete.

Referee #2:

In their paper González Besteiro and colleagues analyse how replication fork progresses when the kinase Chk1 is silenced. They investigate how replication problems affect fork elongation and fork initiation and describe how these are independent events in cells lacking Chk1.
During their investigation they propose a role for the TLS polymerase polη in the bypass of replication barriers and specifically its phosphorylation on Serine 687. This phosphorylation appears to be crucial in modulating how the replication fork elongates when Chk1 is depleted.
Finally they describe how DNA damage persists, even after bypass of replication barriers, and how the fitness of Chk1 depleted cells is regulated.
The paper is well constructed and is timely because it tries to clarify the long running question of the effects caused by Chk1 silencing on fork progression and the control of DNA replication initiation. It analyses the role of polη and its phosphorylation in the process. Overall, while the approach to analyse Chk1 deficient cells by DNA fibres is not entirely novel, the findings the authors provide are interesting and they increase the current knowledge of Chk1 functions in DNA replication. The part of the paper that uncouples origin firing and fork elongation on the fitness of Chk1 silenced cells is well executed and it clarifies many discrepancies present in the current literature.
The authors also discover a new function for the Phosphorylation of Serine 687 of polη that has been only recently identified and whose purpose is still not completely understood.
This is where the paper needs to be improved. The authors clearly think this is one of the main points of their paper as highlighted by the title of the manuscript. I think this work presents data that could be potentially suitable for the EMBO journal but some issues need to be resolved before publication.

Major Issues
1) The authors explain their finding by involving polη in the bypass of replication barriers in cells silenced for Chk1. It is puzzling that silencing of polη does not reduces IdU track length when Chk1 is depleted (Fig3B columns 5 and 6). How are the cells bypassing the replication barriers if polη is such an important player as the authors suggest? I would expect to see a further decrease in IdU track length in that case. Is the IdU pulse too short to see such a difference? If this is the case a longer IdU pulse should be tested, in the same manner as the authors did in fig1 and fig2 of the manuscript.
2) Interestingly, overexpression of polη (Fig4B) does increase the length of IdU tracks when compared to the mock control. The effect is statistically significant but it does look fairly small. Again the result is not straightforward to explain in a cellular background where CDK2 is hyperactivated by silencing of Chk1. In this case, according to the authors' hypothesis, only the mutant S687A should be able to rescue, since it could not be phosphorylated on S687. On the other

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hand, S687D would present the worst case scenario where the phosphomimic is unable to bypass the replication barriers, hence the short IdU tracks. But given the increase in CDK2 activity also WT polη should be hyperphosphorylated and somehow not functional when Chk1 is silenced. Instead WT polη shows the largest rescue on par with S687A. How are the authors explaining this effect? While the authors reasonably pinpoint the effect of polη on S687, they never show if the polymerase is really hyperphosphorylated when Chk1 is silenced. This is only inferred by the result presented in fig 3 but not tested directly. This should be analysed since it is an important part of the authors' model.
3) Another issue regarding polη is the mechanistic role of S687. In various parts of the manuscript the author suggest that S687D affect "DNA synthesis capacity". This is not formally correct as S687 is outside of the catalytic domain of the polymerase and polη mutated in S687A has been shown to be catalytically functional. How do the authors explain the S687D phenotype? What is the phosphorylation of S687 doing? Is the catalytic activity of polη even required for fork elongation when Chk1 is silenced of polη plays a different role? These questions should be answered to improve the reach of the conclusions drawn by the authors.
4) CDC45 silencing does not completely abrogates IdU tracks shortening (fig 7C). Is it worth considering the compounded effect of CDC45 and Roscovitine together?

5) The authors point to non B-DNA as the source of the replication barriers when CDC45 is silenced. This is only inferred as no data is presented showing an increase in non B-form DNA

Minor Issues:
6) The statistics presented in the paper is not really straightforward and makes the figures difficult to evaluate. The significance levels are not presented at all and this should be corrected.
7) In some experiments the authors use a variable number of repeats for the controls and other samples. Only results from complete experiments with matching controls should be presented. (eg Fig3B)
8) The length of IdU tracks when Chk1 is silenced changes a lot in the paper. They go from an average of 3.8um in Fig1C to around 2.5um in Fig3B to 5um S5B. The latter case is in the presence of shRNA instead of siRNA. How are the authors explaining this variability across similar experiments?
9) Fig1 The figure shows an over-analysis of the experiment and some of the plots should be removed or moved to the supplementary.
10) Fig2B Roscovitine only rescues 50% of the track length suggesting that polη is not the only player involved in the bypass of the replication barriers. This should be better discussed.
11) Fig 4E-F the S687D does not present a reduced ability to form foci (see fig S3B) but it seems to change the polymerase retention in the foci (see fig S3C). This in turn could lead to cells showing less foci per nucleus (fig 4E).
12) Fig5 Duplicate plots shown from other figures should be removed.
13) FigS1F Whole cell extracts should be presented to assess if silencing of Chk1 alters the global levels of the proteins analysed.
14) FigS1G Please quantitate the blot. Where is Ubiquitylated PCNA?
15) FigS6B. The siLuc control should be presented in order to assess the levels of Chk1 in normal cells. This is crucial in order to understand the result of the experiment. As of now, we do not know how in excess is Chk1 in the experiment.

Referee #3:

In their work, Gonzalez-Besteiro and colleagues investigated the mechanism underlying the reduced replication fork progression resulting from inhibition or depletion of CHK1 in human cells. They showed that depletion of CHK1 induces replication fork delay not just because of nucleotide shortage deriving from unscheduled origin activation but as a consequence of accumulation of fork barriers, which cannot be overcome by an inhibited poleta. They also demonstrated that, in CHK1 KD cells, such "barriers" are related with persistence of CDC45 because depletion of this protein restores a wild-type phenotype.
The significance of this work is potentially high as there is interest in defining the response to perturbed replication forks or aberrant replication and because CHK1 inhibitors are under evaluation in clinical trials as anticancer drugs. Moreover, although the effect of CHK1 inhibition or depletion

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on replication fork progression/stability has been tested and reported by others, the focus was limited to the role of CHK1 in restraining extra-origin firing through inhibition of CDK2.
Hence, I found the work of interest to EMBOJ readership and potentially adequate for publication, however, I suggest the authors to provide a few additional experimental data on support of their model and hypothesis. Indeed, their observation of fork dynamics, which is the primary level of investigation in the manuscript, might also be explained taking into account different/additional events known to occur in CHK1-depleted cells or possibly occurring in the absence of CHK1. More specifically, the unscheduled activation of MUS81-EME1 following CHK1 inhibition or depletion, as demonstrated by different groups, and subsequent resection or activation of exonucleases at perturbed forks could contribute to the observed phenotype. Thus, specific experiments should be done to evaluate these possibilities.
Moreover, although the data on CDC45's function as barrier are sounding, they are not put in perspective and it is not clear if there is excess CDC45 in the specific experimental system after CHK1 depletion and how excess CDC45 should work as barrier. For instance, is excess CDC45 sufficient to induce barriers in wild-type cells if poleta is depleted?

My suggestions and comments are summarized below:

A general suggestion that applies to every graph. I think that significance is best represented with asterisks or exact p values. The use of letters on the top of each bar or scatter plot confuses a lot, in my opinion.

1) Fig.1. Since the phenotype conferred by CHK1 downregulation is clearly observed only using the 10+30 labelling scheme, I would suggest omitting the data from the 20+20 labelling scheme or showing it as supplementary figure. It would be important, here, to add data excluding that the elongation defect is indeed degradation of nascent strand.

Fig. S1F. I would label the blot as chromatin fraction and I would advice using histones as loading control. Most importantly, how are the authors sure that there is only mono-Ub-PCNA? The point is not trivial, and the author should at least try to check if multiple bands corresponding to poly-Ub-PCNA are accumulating in the absence of CHK1. This might help in excluding that the barrier is related with fork reversal, a process that involves also ZRANB3, which is a poly-Ub-PCNA-binding protein.

Fig. 2C. It seems that replication gets delayed and stops approaching the end of an average-sized replicon. Since CHK1 inactivation also leads to extra origin activation it is likely that the average size of each replicon is reduced and that barriers derives from converging forks. Have the authors evaluated the frequencies of termination events?

Fork asymmetry seems to affect a fraction of the forks. However, the experiment has not been performed using the 10+30 labelling scheme, which is the standard to detect elongation defects. Is there a reason for that? I think that fork asymmetry should be even better represented at increasing labelling periods, making stronger the correlation with the elongation data of Fig. 1.

Fig. 4 B/C. I would suggest reversing order of these panels. In panel 4B, it would be nice to have a rosc control included.

Fig. 6D/E. How the authors reconcile the Comet data (and phosphoH2AX too) with the reported MUS81-dependent formation of DNA breaks after CHK1 depletion or inhibition (see Forment et al 2011; Murfoni et al 2013)?

Fig. 6F/G. I did not find specified if ssDNA has been analysed in nascent or parental strand. Please specify.

Minor points:

I found discussion somehow too speculative.

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Excessive loading of helicase components in Chk1-deficient cell creates replication barriers

Point-by-point responses to Reviewers Concerns

Referee #1:

In this manuscript, Gonzalez Besteiro et al analyze the dynamics of DNA replication following the downregulation of effector checkpoint kinase Chk1, building upon the established notion that Chk1 inhibition promotes extra origin activation and a global reduction in fork speed. The authors use an interesting variation of the standard assay that monitors DNA replication in individual DNA molecules to show that the effect on fork progression in Chk1-deficient cells is not due to reduced fork speed but to a higher frequency of fork stalling events (Figures 1-2). They report PCNA monoubiquitination and the presence of TLS polymerase eta (Pol eta) at forks (Figure S1G; the iPOND experiment, however, indicates that Pol eta is present at forks regardless of Chk1 downregulation). As loss of Chk1 is known to unleash CDK2 activity, roscovitin is added to shChk1-treated cells to partially rescue fork stalling and excess origin firing. Fork progression is also partially rescued by the addition of extra nucleosides, and this effect depends on Pol eta (Figures 3 and S2). Expression of GFP-tagged Pol eta also rescues fork progression to some extent, whereas a phosphomimetic mutant in Ser687 does not, suggesting an inhibitory role for this phosphorylated residue (Figure 4 and S3). The effects of Chk1 on fork rate and origin activation can be separated and are not interdependent, contrary to other reports (Figures 5 and S4). Rescue of fork progression with nucleosides or exogenous Pol eta does not eliminate the DNA damage caused by Chk1 inhibition, whereas reducing the number of active origins by targeting CDC7 or CDT1 does (Figures 6, S5 and S6). Finally, the authors show that targeting CDC45 expression (a component of the CMG helicase) is sufficient to restore normal fork rate, origin firing and cell survival upon loss of Chk1 (Figure 7 and S7). The topic is interesting and the experiments are generally well designed and executed. The study provides several insights into the DNA replication defects caused by the loss of Chk1 activity. I found it difficult, however, to integrate them into a clear central message. I think the article needs to be revised before being considered for publication.

We thank the reviewer for the accurate summary and the interest in our findings. It concerns us that the reviewer finds the message difficult to integrate and we thank the reviewer for giving us the opportunity to improve it. The central message of the manuscript is that Chk1 loss generates replication roadblocks. In this context, the participation of a translesion DNA polymerase such as Polη in DNA elongation represents an important evidence of replication

barrier formation. Such replication barriers slow down replication, independently of the concomitant boost in DNA initiation. Importantly, not only excess origin firing, but also the formation of such replication barriers (but not their bypass) impact on cell growth. We have made modifications throughout the text to improve clarity and hope that the message is now easier to grasp. Below there is a detailed list of the position of each single change, which have been marked in red in the manuscript file.

Abstract: page 2, lines 11-14

Introduction: page 4, lines 17-19

Introduction: page 4, lines 24-26

Results: page 7, lines 24-25

Results: page 8, lines1-3

Results: page 9, lines 5-6

Discussion: page 13, lines 14-16

Discussion: page 14, lines11-12

Discussion: page 17, lines 10-11

We have also modified the title so as not to give disproportionate weight to Polη.

Main comments:

1. There is no conclusive information about the role of Pol eta in the context of Chk1 loss: an inhibitory phosphorylation at Ser687 may prevent its function at bypassing replication barriers (Fig 4), but the relevance of this mechanism is unclear as fork progression can be rescued by Pol eta expression without eliminating DNA damage or cell death (Fig S5). As acknowledged in the Discussion, the phosphorylation at Ser687 may play different functions, e.g. promote or repress the bypass of lesions or barriers, depending on the specific cellular context.

We understand the concern of the reviewer. To further clarify this point we have added Figures 7 and Appendix Figure S8A-B which show that, despite rescuing fork elongation, Polη does not rescue the cellular fitness of Chk1-depleted samples. Hence, Figures 7, EV4 and Appendix Figure S8A-B show that, even when Polη can elongate DNA, such elongation neither prevents DNA damage accumulation nor promotes cell proliferation. Despite the apparent lack of relevance of Polη-dependent elongation to the rescue of the above-mentioned markers, the Polη data served to demonstrate that i) Chk1 loss creates replication barriers, ii) after Chk1 loss, a rescue in fork progression is not sufficient to improve cellular fitness. Such observations are also

important because they led to the identification of the signals that generate Polη substrates after Chk1 loss (dysregulation of helicase loading to DNA). Remarkably, this manuscript demonstrates that the cellular fitness is rescued completely only when replication slow-down is reverted upstream of Polη at the level of the generation of replication barriers (for example by CDC45 depletion) but not downstream replication barrier formation (for example by roscovitine treatment). Hence, while Polη-dependent replication seems irrelevant in the context of Chk1 loss, the data on Polη led to the identification of helicase components as regulators of replication barrier formation and cellular fitness of Chk1-deficient cells.

2. All the data suggest that the main phenotypes caused by loss of Chk1 are derived from an excess in origin firing, which in turn creates "replication barriers" of unknown nature. Can the authors rule out that these barriers are actually conflicts with the transcription machinery? This seems to be the mechanism underlying fork collapse when excess origins are activated by oncogenic stimuli (Macheret and Halazonetis, Nature 2018). The authors may be able to test whether the number of R-loops is elevated, or whether the phenotype of Chk1 loss can be alleviated by an inhibitor of transcription elongation.

The reviewer brings up a good point. We have performed DNA fiber assays and γH2AX immunofluorescence/western blot with DRB, and inhibitor of transcription elongation (Figure EV2B). Our new data rule out that R-loops account for fork slow-down in Chk1-depleted cells. Moreover, R-loops do not underlie γH2AX accumulation in Chk1-inhibited cells (Figure 1-see below), which correlates with DSB accumulation downstream of excess origin firing (Figure 6). We have also tested other potential sources for replication barriers such as fork reversal (Figure EV2E) and MUS81-dependent DSBs (Figure EV2C), as suggested by reviewer 3. Hence, thanks to reviewers´ comments we were able to further characterize the replication roadblocks generated by Chk1 loss which involve excess recruitment of helicase factors to DNA.

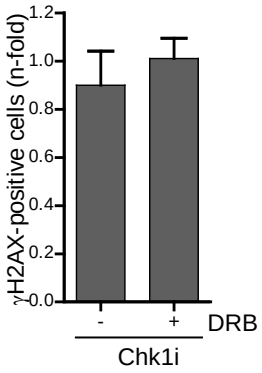

Figure 1: Conflicts with transcription are not the cause of pannuclear γH2AX accumulation in Chk1-inhibited cells.

U2OS cells were treated for 5 hours with the Chk1 inhibitor Go6976, in the presence or not of 100 μM DRB, an inhibitor of transcription elongation. The data are shown as the mean+SD of 2 independent experiments.

3. It is clear that downregulation of Cdc45 partially rescues the phenotypes caused by Chk1 inactivation because it reduces the percentage of active origins. However, the concept that

"replication barriers are created by excess CDC45" (Discussion, first paragraph) is an overstatement. No evidence is provided to support that an excess of CDC45 creates non-B DNA structures. Even the notion that CDC45 is bound to DNA in excess following Chk1 downregulation relies on observations from other groups and should be independently confirmed in the authors' cellular system. The experiment shown in Figure 7E shows total levels of CDC45, not chromatin-bound levels, and siLuc and siChk1 are shown in different panels.

We agree that "replication barriers are created by excess CDC45" is an overstatement, so we have accordingly modified the sentence (page 14, lines 11-12). We do provide evidence however that our data in Figure 8 cannot be solely due to CDC45-dependent control of origin firing: a) roscovitine, which normalizes DNA initiation without removing replication barriers, partially rescues cellular fitness of Chk1-depleted cells; b) the downregulation of CDC45, which normalizes DNA initiation and reduces the amount of Polη-sensitive replication barriers, completely rescues cell survival in Chk1-depleted cells. We have obtained similar results after downregulation of another helicase component, MCM2. Also in this case, a reduction in the amount of Polη-sensitive replication barriers was accompanied by an improvement in the rescue of cellular fitness in comparison to Roscovitine (Figure EV5 in the revised version of the manuscript).

To directly address the reviewer´s concerns, we have performed a western blot showing that excess CDC45 is present in the insoluble/chromatin fraction of Chk1-depleted cells (Figure 8A). We have even included a Roscovitine treatment, which, although sufficient to restore origin firing levels (Figure 3), was insufficient to normalize the levels of insoluble CDC45 in Chk1-deficient cells (Figure 8A). This is consistent with the hypothesis that excess CDC45 creates the substrate for Polη-dependent replication. The experiment showing total levels of CDC45 is now part of Figure 8C; as requested, siLuc and siChk1 are now shown in a single panel.

Other minor points:

1. p3 (Introduction), the sentence "In unperturbed conditions, Chk1 loss triggers excess origin firing and reduced rates of fork elongation" should also cite earlier work from the Lukas-Bartek lab (Syljuasen et al MCB 2005).

We thank the reviewer for spotting that this reference was missing. We have now included it (page 3 line 15).

2. p8, l4, "pulses of IdU (10-100m)" should read "(10-80m)"

Thank you for mentioning this mistake, we have now corrected it (page 6 line 6).

3. p15, l7 from bottom, the presence of ssDNA is referred to as a marker of "replication catastrophe". This term might have been used in other papers, but it seems more reasonable to use "replication stress".

We agree that replication stress is a more conservative statement which more accurately reflects the meaning of ssDNA accumulation. We have made the corresponding amendments (page 11 line 19).

4. p20, the sentence "The DNA fiber assay is insensitive to such localized sites..." is not clear.

We apologize for not being clear. We have rewritten this sentence (page 14, lines 7-8).

5. Reference Rodriguez-Acebes et al (JBC 2018) is incomplete.

Thank you for spotting this mistake, we have now corrected it.

Referee #2:

In their paper González Besteiro and colleagues analyse how replication fork progresses when the kinase Chk1 is silenced. They investigate how replication problems affect fork elongation and fork initiation and describe how these are independent events in cells lacking Chk1. During their investigation they propose a role for the TLS polymerase polη in the bypass of replication barriers and specifically its phosphorylation on Serine 687. This phosphorylation appears to be crucial in modulating how the replication fork elongates when Chk1 is depleted. Finally they describe how DNA damage persists, even after bypass of replication barriers, and how the fitness of Chk1 depleted cells is regulated. The paper is well constructed and is timely because it tries to clarify the long running question of the effects caused by Chk1 silencing on fork progression and the control of DNA replication initiation. It analyses the role of polη and its phosphorylation in the process. Overall, while the approach to analyse Chk1 deficient cells by DNA fibres is not entirely novel, the findings the authors provide are interesting and they increase the current knowledge of Chk1 functions in DNA replication. The part of the paper that uncouples origin firing and fork elongation on the fitness of Chk1 silenced cells is well executed and it clarifies many discrepancies present in the current literature. The authors also discover a new function for the Phosphorylation of Serine 687 of polη that has been only recently identified and whose purpose is still not completely understood. This is where the paper needs to be improved. The authors clearly think this is one of the main points of their paper as highlighted by the title of the manuscript. I think this work presents data that could

be potentially suitable for the EMBO journal but some issues need to be resolved before publication.

We thank the reviewer for the kind comments. We notice that Reviewer 2 is mostly interested in the mechanistic regulation of Polη phosphorylation. Although we have specifically addressed the issues raised by the reviewer, we believe that the fact that reviewer 2 focused on this aspect of our research may relate to the writing defects pointed out by reviewer 1 (which had been amended in the current version of our manuscript). In this respect, we would like to point out that the molecular mechanisms by which Polη phosphorylation impedes DNA synthesis in Chk1-depleted cells is not the main focus of our study. Instead, and as stated before, our data on Polη provided a tool to identify replication roadblocks and evaluate the impact of fork progression on biologically relevant outputs.

Major Issues

1) The authors explain their finding by involving polη in the bypass of replication barriers in cells silenced for Chk1. It is puzzling that silencing of polη does not reduces IdU track length when Chk1 is depleted (Fig3B columns 5 and 6). How are the cells bypassing the replication barriers if polη is such an important player as the authors suggest? I would expect to see a further decrease in IdU track length in that case. Is the IdU pulse too short to see such a difference? If this is the case a longer IdU pulse should be tested, in the same manner as the authors did in fig1 and fig2 of the manuscript.

Our data show that Chk1 creates replication barriers which can be, but are not, bypassed by Polη. There are three ways to promote Polη-dependent bypass of replication barriers: a) removal of the inhibitory phosphorylation of CDK2 by roscovitine (Figure 3B); b) overexpression of GFP-Polη (Figures 4D, EV3A and Appendix Figure S4); c) supplementation with nucleosides (Appendix Figure S3B-C). Hence, despite its recruitment to replication forks (Figures EV2G and EV3C-D), endogenous Polη is limited in its capacity to elongate forks devoid of Chk1. And that is why silencing Polη does not provoke a further slow-down of forks in Chk1-depleted cells.

2) Interestingly, overexpression of polη (Fig4B) does increase the length of IdU tracks when compared to the mock control. The effect is statistically significant but it does look fairly small. Again the result is not straightforward to explain in a cellular background where CDK2 is hyperactivated by silencing of Chk1. In this case, according to the authors' hypothesis, only the mutant S687A should be able to rescue, since it could not be phosphorylated on S687. On the other hand, S687D would present the worst case scenario where the phosphomimic is unable to bypass the replication barriers, hence the short IdU tracks. But given the increase in CDK2

activity also WT polη should be hyperphosphorylated and somehow not functional when Chk1 is silenced. Instead WT polη shows the largest rescue on par with S687A. How are the authors explaining this effect? While the authors reasonably pinpoint the effect of polη on S687, they never show if the polymerase is really hyperphosphorylated when Chk1 is silenced. This is only inferred by the result presented in fig 3 but not tested directly. This should be analysed since it is an important part of the authors' model.

We can see why the data on GFP-Polη overexpression may cause confusion. We have performed an experiment to shed light on this issue. The experiment consisted in evaluating the ability of decreasing amounts of GFP-Polη-WT to rescue IdU track lengths of Chk1-depleted cells. As observed in the new Figure EV3, GFP-Polη is overexpressed several folds in comparison to endogenous Polη. As the amount of exogenous GFP-Polη decreases, GFP-Polη-WT loses its ability to rescue track length. However, the ability of GFP-Polη-S687A to rescue track length remains intact (Figure EV3). We thus believe that overexpression overrides phosphorylation.

Even in the face of these results, and because it was technically unfeasible to determine S687 phosphorylation of endogenous Polη, we tested global changes in the phosphorylation of GFP-Polη in XP30RO cells, which over-express GFP-Polη just 2-3 folds. By using 2D gel experiments, we found that GFP-Polη is hyper-phosphorylated in Chk1-depleted XP30RO cells (Appendix Figure S2). While such an observation provides incomplete answers to this reviewer´s request, we hope that the integration of this result with the data on GFP-Polη mutants (Figures 4 and EV3) fulfills the requests regarding Polη regulation after Chk1 downregulation.

3) Another issue regarding polη is the mechanistic role of S687. In various parts of the manuscript the author suggest that S687D affect "DNA synthesis capacity". This is not formally correct as S687 is outside of the catalytic domain of the polymerase and polη mutated in S687A has been shown to be catalytically functional. How do the authors explain the S687D phenotype? What is the phosphorylation of S687 doing? Is the catalytic activity of polη even required for fork elongation when Chk1 is silenced of polη plays a different role? These questions should be answered to improve the reach of the conclusions drawn by the authors.

We appreciate this comment and apologize for the lack of clarity. We did not intend to suggest that S687 phosphorylation alters the catalytic activity of Polη. We instead proposed that CDK-dependent Polη phosphorylation in Chk1-deficient cells impedes Polη polymerase function, altering neither its recruitment to forks nor its catalytic activity. We believe that further exploration of the molecular mechanism is beyond the scope of this manuscript. Nevertheless, we have performed experiments to address this specific issue raised by the reviewer. As shown

in new Appendix Figure S4A, GFP-Polη-dependent fork elongation in Chk1-depleted cells requires its catalytic activity. Moreover, this point is addressed in the discussion (page 15, lines 1-8) and mentioned in the results sections (page 8, lines 9-10). In addition, we have modified the term "DNA synthesis capacity" and used "rates of replication barrier bypass" instead (page 8, line 22).

4) CDC45 silencing does not completely abrogates IdU tracks shortening (fig 7C). Is it worth considering the compounded effect of CDC45 and Roscovitine together?

This is an interesting point. We propose that excess CDC45 creates DNA replication barriers whose bypass is hampered by high CDK activity. If that is the case, Roscovitine should not further rescue the elongation when replication barriers are removed (siCDC45). We have hence performed the experiment suggested by the reviewer and, in agreement with our predictions, Roscovitine did not further lengthen IdU tracks after CDC45 depletion in a Chk1-deficient background. We have included the results in Figure 8B.

5) The authors point to non B-DNA as the source of the replication barriers when CDC45 is silenced. This is only inferred as no data is presented showing an increase in non B-form DNA.

As the reviewer indicates, the concept that non-B form DNA increases after Chk1 depletion is inferred from our data as we cannot directly measure non-B DNA. During this revision we have realized that there might be alternative sources of replication barriers. For example, the data obtained when depleting MCM2, another helicase component (Figure EV5), suggest that excessive helicase complexes bound to DNA could act as a barrier. Hence, non B-DNA is not the only potential source of replication barriers. Moreover, and according to Reviewer 1´s and 3´s comments, we have now included data exploring R-loops, MUS81-dependent DSBs and converging forks as sources of the replication barriers that accumulate in Chk1-deficient cells (Figure EV2). We have modified the discussion accordingly. See page 14, lines 11-12 and page 15 lines 9-19.

Minor Issues:

6) The statistics presented in the paper is not really straightforward and makes the figures difficult to evaluate. The significance levels are not presented at all and this should be corrected.

While we are of course ready to change all plots in the manuscript to the asterisk-type of analysis we would like to have the chance to debate further with the reviewers and the journal on the advantages of the statistical method presented in this manuscript. If, after reading this

*letter, the reviewers still consider it necessary to modify the statistical analysis, we will certainly change it in all figures of the manuscript. The use of asterisks has an advantage because p-values are showed. However, when increasing the number of samples, only a selected subset of one-to-one comparisons can be shown. Moreover, the high number of square brackets makes it sometimes very difficult to comprehend which samples are not significantly different (which is the most relevant information that should be deduced from the statistical analysis). The letter-type of analysis, on the other hand, group samples which are not significantly different between each other. Hence, all samples containing an "a" are not significantly different between each other but are significantly different from all samples having no "a". Thus, if two samples do not share any letter, they are significantly different. We firmly believe that this way of presenting the statistics allows a simple and fast comparison among **all** samples, which is usually not the case when the focus is made on the difference between 2 samples. In the revised version of the manuscript, both the Materials and Methods section and the figure legends were modified to improve the explanation (page 22, lines 19-21 and page 29, lines 19-21). The p-value cut-offs are also specified (page 28, lines 21-22).*

Finally, we would also like to stress the fact that denoting significance with letters is a common practice in other fields and a good option if many conditions are being compared. To illustrate our thoughts, we show below one graph in which the two ways of visualizing the statistics have been applied. We hope that the reviewer is willing to respect our views on this issue.

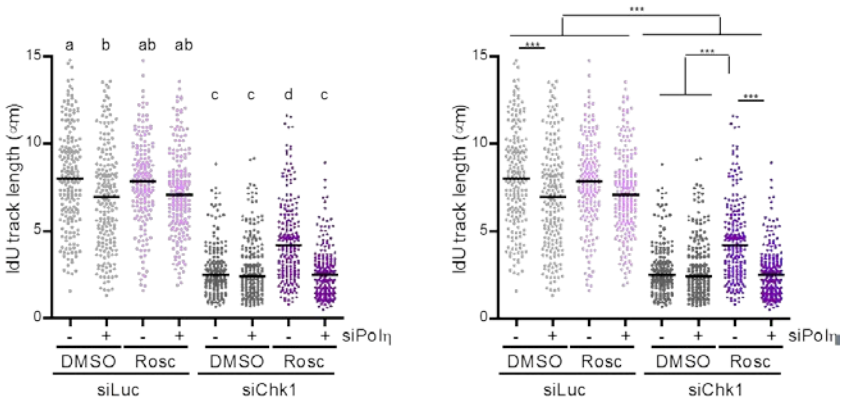

Figure 2: Comparison between two ways of visualizing statistics.

7) In some experiments the authors use a variable number of repeats for the controls and other samples. Only results from complete experiments with matching controls should be presented. (eg Fig3B) .

Figures 3B, 5B and Appendix Figure S3C have been modified to satisfy this request.

8) The length of IdU tracks when Chk1 is silenced changes a lot in the paper. They go from an average of 3.8um in Fig1C to around 2.5um in Fig3B to 5um S5B. The latter case is in the presence of shRNA instead of siRNA. How are the authors explaining this variability across similar experiments?

We have checked the IdU track lengths throughout the paper. We noticed that the outlier (in terms of IdU track lengths after Chk1 depletion) is Figure EV1; in all other figures track lengths are in the range of 2.5-3 μm, which we believe is acceptable. Data in Figure EV1 were obtained with a different batch of siRNA, which was probably more inefficient. In the case of Figure S5B (current Figure EV4A), differences between siRNA and shRNA can be easily explained by differential ability to silence Chk1.

9) Fig1 The figure shows an over-analysis of the experiment and some of the plots should be removed or moved to the supplementary.

Figure 1 has been modified to fulfill reviewers 2 and 3 requests.

10) Fig2B Roscovitine only rescues 50% of the track length suggesting that polη is not the only player involved in the bypass of the replication barriers. This should be better discussed.

We agree with this comment. Although this idea was mentioned in the original version of the manuscript, we have now strengthened the discussion on this issue (page 15, lines 9-19).

11) Fig 4E-F the S687D does not present a reduced ability to form foci (see fig S3B) but it seems to change the polymerase retention in the foci (see fig S3C). This in turn could lead to cells showing less foci per nucleus (fig 4E).

We agree with this comment. Indeed, the data on Figures S3C and 4E (current Figures EV3B and 4E) was obtained after CSK extraction, as mentioned in the main text (page 8, pages 24-26). We have included this detail in the legend of the Figure 4E because it was missing (page 31, line 1).

12) Fig5 Duplicate plots shown from other figures should be removed.

The necessary adjustments have been made to satisfy this request.

13) FigS1F Whole cell extracts should be presented to assess if silencing of Chk1 alters the global levels of the proteins analysed.

The whole cell extracts blots have been added as requested (Figure EV2F).

14) FigS1G Please quantitate the blot. Where is Ubiquitylated PCNA?

We have quantified the iPOND and added the result to the corresponding figure. Unfortunately, when the iPONDs were performed, we had not contemplated ubiquitylated PCNA, so the membrane was cut around 32 kDa, which allows detection of PCNA but not of ubiquitylated PCNA.

15) FigS6B. The siLuc control should be presented in order to assess the levels of Chk1 in normal cells. This is crucial in order to understand the result of the experiment. As of now, we do not know how in excess is Chk1 in the experiment.

The requested control has been added (current Figure EV4D-E).

Referee #3:

In their work, Gonzalez-Besteiro and colleagues investigated the mechanism underlying the reduced replication fork progression resulting from inhibition or depletion of CHK1 in human cells. They showed that depletion of CHK1 induces replication fork delay not just because of nucleotide shortage deriving from unscheduled origin activation but as a consequence of accumulation of fork barriers, which cannot be overcome by an inhibited poleta. They also demonstrated that, in CHK1 KD cells, such "barriers" are related with persistence of CDC45 because depletion of this protein restores a wild-type phenotype. The significance of this work is potentially high as there is interest in defining the response to perturbed replication forks or aberrant replication and because CHK1 inhibitors are under evaluation in clinical trials as anticancer drugs. Moreover, although the effect of CHK1 inhibition or depletion on replication fork progression/stability has been tested and reported by others, the focus was limited to the role of CHK1 in restraining extra-origin firing through inhibition of CDK2. Hence, I found the work of interest to EMBOJ readership and potentially adequate for publication, however, I suggest the authors to provide a few additional experimental data on support of their model and hypothesis. Indeed, their observation of fork dynamics, which is the primary level of investigation in the manuscript, might also be explained taking into account different/additional events known to occur in CHK1-depleted cells or possibly occurring in the absence of CHK1. More specifically, the unscheduled activation of MUS81-EME1 following CHK1 inhibition or depletion, as demonstrated by different groups, and subsequent resection or activation of exonucleases at perturbed forks could contribute to the observed phenotype. Thus, specific experiments should be done to evaluate these possibilities. Moreover, although the data on CDC45's function as barrier are sounding, they are not put in perspective and it is not clear if there is excess CDC45 in the specific experimental system after

CHK1 depletion and how excess CDC45 should work as barrier. For instance, is excess CDC45 sufficient to induce barriers in wild-type cells if poleta is depleted?

We thank the reviewer for acknowledging the significance of our work and bringing up important issues. To evaluate the contribution of MUS81 and Mre11 to the observed fork dynamics phenotypes, we have performed DNA fiber analysis with siMUS81 and mirin (see below). Moreover, and as suggested by reviewer 1, we have explored the contribution of R-loops to the phenotype we observe. These data have been included in Figure EV2.

Regarding CDC45, two previous reports have shown that the sole overexpression of CDC45 induces fork asymmetry. These references were actually included in the original version of the manuscript (page 12, lines 14-15). In the revised version, we have included a western blot showing that excess CDC45 is present in the insoluble/chromatin fraction of Chk1-depleted cells (Figure 8C), which is in agreement with previous reports (Syljuassen, MCB, 2005; Zuazua-Villar, NAR, 2015). Moreover, we have also tested the effect of downregulating MCM2, another component of the replicative helicase. Reminiscent of CDC45 knock-down, MCM2 down-modulation alleviated the replication fork slow-down provoked by Chk1 loss independently of Polη. The data have been included in Figure EV5. Thus, the nature of the replication barrier we describe might be related to excess or tight helicase-DNA complexes. Alternatively, as addressed in the discussion (page 14, lines 11-21), excessive firing of neighbor origins, in contrast to the activation on new replication factories, might account for the generation of replication fork obstacles in Chk1-deficient cells.

My suggestions and comments are summarized below:

A general suggestion that applies to every graph. I think that significance is best represented with asterisks or exact p values. The use of letters on the top of each bar or scatter plot confuses a lot, in my opinion.

We understand the reviewer´s concern and we very much respect his/her opinion. We are ready to fulfill the request but we would like to present arguments in favor of the letter type of analysis. We copy below the arguments presented to reviewer 2.

While we are of course ready to change all plots in the manuscript to the asterisk-type of analysis we would like to have the chance to debate further with the reviewers and the journal on the advantages of the statistical method presented in this manuscript. If, after reading this letter, the reviewers still consider it necessary to modify the statistical analysis, we will certainly change it in all figures of the manuscript. The use of asterisks has an advantage because p-values are showed. However, when increasing the number of samples, only a selected subset of

one-to-one comparisons can be shown. Moreover, the high number of square brackets makes it sometimes very difficult to comprehend which samples are not significantly different (which is the most relevant information that should be deduced from the statistical analysis). The letter-type of analysis, on the other hand, group samples which are not significantly different between each other. Hence, all samples containing an "a" are not significantly different between each other but are significantly different from all samples having no "a". Thus, if two samples do not share any letter, they are significantly different. We firmly believe that this way of presenting the statistics allows a simple and fast comparison among ***all*** *samples, which is usually not the case when the focus is made on the difference between 2 samples. In the revised version of the manuscript, both the Materials and Methods section and the figure legends were modified to improve the explanation (page 22, lines 19-21 and page 29, lines 19-21). The p-value cut-offs are also specified (page 28, lines 21-22).*

Finally, we would also like to stress the fact that denoting significance with letters is a common practice in other fields and a good option if many conditions are being compared. To illustrate our thoughts, we show in Figure 2 of this letter one graph in which the two ways of visualizing the statistics have been applied. We hope that the reviewer is willing to respect our views on this issue.

1) Fig.1. Since the phenotype conferred by CHK1 downregulation is clearly observed only using the 10+30 labelling scheme, I would suggest omitting the data from the 20+20 labelling scheme or showing it as supplementary figure. It would be important, here, to add data excluding that the elongation defect is indeed degradation of nascent strand.

In response to this request, we have performed DNA fiber analysis of Chk1-depleted cells treated with mirin, an inhibitor of the Mre11 exonuclease, which degrades nascent DNA. Mre11 inhibition did not alleviate the elongation defect of Chk1-depleted cells. We used UV-induced Mre11-dependent degradation in a Rad51-defective background as a positive control. Such results are showed in Figure EV2A. Also as suggested by reviewers 2 and 3, we have modified Figure 1.

Fig. S1F. I would label the blot as chromatin fraction and I would advice using histones as loading control. Most importantly, how are the authors sure that there is only mono-Ub-PCNA? The point is not trivial, and the author should at least try to check if multiple bands corresponding to poly-Ub-PCNA are accumulating in the absence of CHK1. This might help in excluding that the barrier is related with fork reversal, a process that involves also ZRANB3, which is a poly-Ub-PCNA-binding protein.

As suggested by the reviewer, we have relabeled the blot (now Figure EV2F). Regarding the loading control, we believe the nucleolar protein fibrillarin is a valid control because it is undetectable in the soluble fraction (Figure 3-see below). Notwithstanding this, we have used H2B as a control in the western blot of chromatin fraction shown in Figure 8A, which has been added to the manuscript during this revision. Regarding fork reversal, by making use of the PARP-1 inhibitor olaparib and a siRNA targeting RecQ1 (Berti et al., Nat Struct Mol Biol, 2013), we conclude that fork reversal takes place in Chk1-depleted cells. Because fork reversal is a common mechanism elicited upon fork stalling, we were not surprised by these results. We believe that the fork reversal data reinforce the notion that Chk1 loss creates replication barriers. These data are showed in Figure EV2E of the current version of the manuscript and discussed (page 15 lines 13-19).

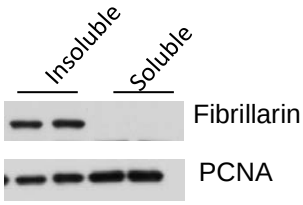

Figure 3: Fibrillarin marks the insoluble fraction in CSK extraction experiments.

U2OS cells were subjected to a CSK extraction (0.5% Triton, 5 min) and protein samples analyzed by Western Blot.

Fig. 2C. It seems that replication gets delayed and stops approaching the end of an average-sized replicon. Since CHK1 inactivation also leads to extra origin activation it is likely that the average size of each replicon is reduced and that barriers derives from converging forks. Have the authors evaluated the frequencies of termination events?

This is an interesting point. We evaluated the frequency of termination events and found that they are reduced upon Chk1 depletion (Figure EV2D). Thus, even if origin firing is induced, replication fork progression is so slow that forks take longer to converge.

Fork asymmetry seems to affect a fraction of the forks. However, the experiment has not been performed using the 10+30 labelling scheme, which is the standard to detect elongation defects. Is there a reason for that? I think that fork asymmetry should be even better represented at increasing labelling periods, making stronger the correlation with the elongation data of Fig. 1.

In the DNA combing experiment, total track lengths (CldU+IdU) from forks that emerge from the same origin are measured. The differences unveiled in Figure 1 resulted from the comparison between CldU and IdU lengths. But the total fork length in a 10+30 or 20+20 setting is equal, because the total labeling time is equal (40 minutes). Thus, because in the combing protocol total fork lengths are the key variables (total fork length at one side of the

origin is compared with the total fork length at the other side of the origin), the result would not change if we used a 10+30 labeling scheme. We do not know why the results in Figure2D are not as dramatic as one would expect from Figure 1 but we noticed that they were performed with a batch of siRNA that did not downregulate Chk1 as well as the batch used for most figures. In fact, one can observe that the difference in track length between siLuc and siChk1 samples in Figure2D (lower right panel) are not as profound as those obtained in other experiments (e.g. Appendix Figures S1 and S3). We have still chosen to include these data because the asymmetry is observed and the technique is valuable as, in the literature, DNA combing is considered as the definite proof of fork asymmetry.

Fig. 4 B/C. I would suggest reversing order of these panels. In panel 4B, it would be nice to have a rosc control included.

According to this suggestion, we have modified the order of the panels in Figure 4. Regarding the Rosc control, we feel that including it in Figure 4 of the manuscript will affect the symmetry of this figure. However, we show below an experiment confirming that Rosc and GFP-Polη overexpression function in an epistatic fashion.

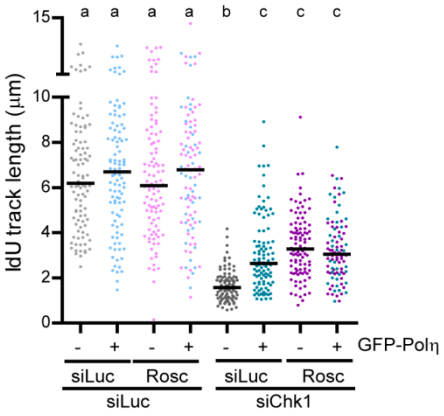

Figure 4: Roscovitine and GFP-Polη overexpression rescue fork slow-down in Chk1-depleted cells to a similar extent and show an epistatic interaction.

Fig. 6D/E. How the authors reconcile the Comet data (and phosphoH2AX too) with the reported MUS81-dependent formation of DNA breaks after CHK1 depletion or inhibition (see Forment et al 2011; Murfoni et al 2013)?

We do not quite understand the conflict brought up by this comment. Our data show that excess origin firing results in DSBs and γH2AX accumulation, but do not exclude a role of Mus81. Indeed, we have confirmed the data to which the reviewer refers to, e.g. that Chk1 depletion causes Mus81 dependent DSB accumulation (Figure 5-see below). What our data may challenge is the interpretation of those results. While the current model proposes that MUS81 cuts stalled forks in a Chk1-deficient background, this has not been conclusively shown.

Our data go against this interpretation because we show that fork stalling is not related to DNA damage accumulation (Figures 6 and EV4). Our data favor the conclusions reached by Toledo et al., Cell, 2013, which point to a central role of RPA levels in genome integrity. In particular, we believe that surplus DNA initiation perhaps depletes RPA at a global scale, leading to MUS81-dependent fork breakage in all active replication factories (irrespective of whether they are stalled or not). Instead, fork slow-down is a local effect related to the accumulation of replication barriers, which: i) takes place even after origin firing levels have been restored; ii) does not induce MUS81-mediated cleavage.

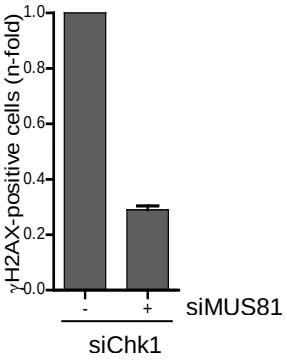

Figure 5: MUS81 downregulation impedes the accumulation of pannuclear γH2AX in Chk1-inhibited cells.

U2OS cells were transfected with siRNA against Chk1 and MUS81, as indicated. The data are shown as the mean+SD of 2 independent experiments. In siLuc-treated samples, no pannuclear γH2AX was detected (not shown).

Fig. 6F/G. I did not find specified if ssDNA has been analysed in nascent or parental strand. Please specify.

The reviewer is right. We had mentioned in the Materials and Methods section that BrdU was added for 36 hours, which implies that parental ssDNA was analyzed. We have now specified this in the Materials and Methods section (page 19 line 21) and in the figure legend (page 32, line 12).

Minor points:

I found discussion somehow too speculative.

We have modified the discussion taking into consideration the points raised by all reviewers and removed the paragraphs which contained speculations. Below there is list of the sentences that have been removed.

" Regarding CDT1 and CDC7, we predict that a more drastic reduction in the levels of these factors would yield similar results as those obtained by CDC45/MCM2 depletion. Indeed, others have worked under conditions in which CDC7 inactivation restores reduced DNA elongation of Chk1-deficient cells (Petermann et al, 2010; Rodriguez-Acebes et al, 2018)."

"formation of non-B DNA via a function beyond its canonical role in DNA replication initiation. For example, CDC45 could regulate the coupling between the polymerase and the helicase, thus

promoting the aberrant folding of ssDNA between the polymerase and the helicase in case of uncoupling."

"Our work is the first one that demonstrates the in vivo relevance of Polη S687 phosphorylation to the synthesis of naturally occurring structured DNA"

"Unexpectedly, we found that low NS availability, characteristic of Chk1-deficient cells (Buisson et al, 2015; Techer et al, 2016), restricts Polη-dependent DNA synthesis. This is in line with the fact that Polη has a lower affinity for nucleotides than replicative polymerases (Washington et al, 2003). We conclude that low NS levels and high CDK activity are needed to keep Polη inactive."

"Although a drastic downregulation of CDC7 or CDT1 plausibly impacts on nascent DNA elongation (see above), the same conditions that reveal origin-fork speed uncoupling in Chk1-depleted backgrounds, reveal coupling in Wee1-depleted cells."

We also reduce the speculation in certain paragraphs making them more factual

1) "Thus, the fact that Polη overexpression alleviates slow elongation in Chk1-deficient cells implies the presence of non-B DNA all along the genome. Therefore, Chk1 inactivation might create secondary structures in DNA that recruit and can be bypassed by Polη.

for

"Thus, our observation that Polη overexpression alleviates slow elongation in Chk1-deficient cells implies the presence of replication barriers all along the genome."

2) "We thus favor the hypothesis that CDK2-mediated Polη phosphorylation at S687 impedes the bypass of structured DNA created by Chk1 loss. In apparent contrast, Polη phosphorylation at S687 promotes the bypass of UV-damaged DNA (Bertoletti et al, 2017; Dai et al, 2016), even if Polη catalytic activity is unaffected by phosphorylation (Bertoletti et al, 2017). "

for

"We thus favor the hypothesis that CDK2-mediated Polη phosphorylation at S687 regulates the bypass of replication obstacles. The outcome may vary depending on the obstacle: negative after Chk1 loss but positive after UV irradiation (Bertoletti et al, 2017; Dai et al, 2016)"

3) "Our data show that excess CDC45, but not high CDK activity, contributes to the adoption of non B DNA conformations by DNA in Chk1-depleted cells"

for

"Our data also show that excess CDC45/MCM2, but not high CDK activity, contributes to the generation of the replication intermediates that recruit Pol η in Chk1-depleted cells."

2nd Editorial Decision 28th May 2019

Thank you for your patience during our re-review of your revised manuscript. I have now had a chance to carefully go through it and check it from the editorial side, and we have also received comments from two of the original referees, copied below for your information. Since both reviewers are generally satisfied with the revisions, we shall be happy to publish the study in The EMBO Journal, pending addressing of a few remaining specific points. In particular, both referees raise several concerns with presentation and interpretation that would need to be addressed by textual changes, including alterations to the title (see referee 1), which I feel could be both more accurate and more widely accessible. In addition, I think the study should benefit from inclusion of some additional immunoblot controls as requested by referee 1, pt 3, and by considering referee 3's issue 4 about reproducibility and presentation of the CHK1 knockdown experiments.

REFERE REPORTS

Referee #1:

V. Gottifredi and coworkers have addressed most of my comments to the earlier version of the manuscript. I appreciate their efforts towards answering my three main questions as well as several minor points. My general opinion about the manuscript is positive but I would like to pass along a few comments to the Editor and authors.

1. I am surprised by the change of Title. I had previously commented about having some difficulty to grasp the "central message" of the paper. The authors state in the rebuttal letter that this should be that "Chk1 loss generates replication roadblocks"; possibly the new Title aims at emphasizing it. However, the study describes a rather complex mechanistic network in which the loss of Chk1 drives two independent effects: one on fork progression (mediated by roadblocks) and another one on the abnormal frequency of origin activation. It is mentioned several times that both effects compromise the fitness of Chk1-deficient cells: roadblocks affect cell viability but the excessive origin firing triggers DNA damage. I believe that a Title that reflected this duality would be a more accurate representation of the study. The emphasis of the current title is placed on "excessive loading of helicase components", which is technically tricky as the term "excessive loading" is not synonymous with having "higher amounts on chromatin". For instance, cells could have loaded the same amounts of CDC45 and then failed to unload it in a timely manner in S phase due to the altered replication dynamics.

2. My comment about the possibility of replication-transcription conflicts has been nicely addressed and is now part of a set of data covering also other possibilities raised by the other reviewers.

3. My third question was about showing the actual amounts of CDC45 protein on chromatin, rather than total levels. This becomes a key point considering the orientation that the authors give to the discussion (and the new title). A new experiment in Figure 8A addresses the question, but this could have been completed with a few additional western blots directed to other helicase components such as MCM2-7 and/or GINS proteins. The conclusion that higher amounts of helicase components on chromatin cause the roadblocks could be made stronger with a few immunoblots of little technical complexity (at least the MCM proteins are very abundant and antibodies can be obtained from many laboratories or commercial sources). It is of course possible that only CDC45 is responsible for the effect, but the experiment in Figure EV5 with downregulation of MCM2 (no immunoblot shown) suggests otherwise.

Referee #3:

I read with interest the revised version of the work and I appreciated the impressive amount of new data added by the authors.
I carefully checked the answers provided to my comments and to those raised by the other reviewers

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and I think that the additional data as well as the authors' explanations are satisfactory.
In principle, I would recommend the work for publication, however, I have still some concerns with few data and some minor comments I hope the authors can deal with.

1. I thank the authors very much for the detailed explanation on their preference about representation of statistical significance marking on graphs. I still would suggest to opt for the other way but I respect the choice of the authors;

2. Concerning the nature of "roadblock", I would suggest to tone down the claim about the presence of reversed forks as there is not formal evaluation of them. In addition, I see that pre-treatment with Olaparib increases the slow-replicating phenotype (Fig. EV2E). One could argue that also formation of reversed forks contributes to replication slow-down and not just that reversed forks are formed upon CHK1 loss. That is, reversed forks are the barriers. Indeed, formation of reversed forks slow-down replication. The authors should include this possibility in their discussion, at least;

3. As far as the role of MUS81 goes, I welcome the possible explanation of the authors about how MUS81-dependent DSBs can form upon CHK1 loss or inhibition. However, as the authors added the experiment with MUS81 RNAi in Fig. EV2, why do not mention the panel? Indeed, only panel EV2F-G are mentioned in the text. In addition, I think it would be fair to mention the works reporting MUS81-dependent DSBs in CHK1-deficient cells;

4. An important point concerns the sub-optimal performance of the siCHK1 batch used in the experiments shown in Fig. 2D. I'm sorry but if they know that the result may be different because of the batch variability it would be better to repeat the data using more efficient reagents or to move the data as a supplementary material adding a panel with the control of CHK1 depletion so that readers can appreciate that.

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Chk1 loss creates replication barriers that compromise survival independently of origin firing levels

Point-by-point responses to Reviewers Concerns

Referee #1:

V. Gottifredi and coworkers have addressed most of my comments to the earlier version of the manuscript. I appreciate their efforts towards answering my three main questions as well as several minor points. My general opinion about the manuscript is positive but I would like to pass along a few comments to the Editor and authors.

1. I am surprised by the change of Title. I had previously commented about having some difficulty to grasp the "central message" of the paper. The authors state in the rebuttal letter that this should be that "Chk1 loss generates replication roadblocks"; possibly the new Title aims at emphasizing it. However, the study describes a rather complex mechanistic network in which the loss of Chk1 drives two independent effects: one on fork progression (mediated by roadblocks) and another one on the abnormal frequency of origin activation. It is mentioned several times that both effects compromise the fitness of Chk1-deficient cells: roadblocks affect cell viability but the excessive origin firing triggers DNA damage. I believe that a Title that reflected this duality would be a more accurate representation of the study. The emphasis of the current title is placed on "excessive loading of helicase components", which is technically tricky as the term "excessive loading" is not synonymous with having "higher amounts on chromatin". For instance, cells could have loaded the same amounts of CDC45 and then failed to unload it in a timely manner in S phase due to the altered replication dynamics.

The title has been changed according to reviewer´s 1 request. We have also included a missing reference, Kurashima et al, 2018, which highlights the link between CDC45 and Pol η.

2. My comment about the possibility of replication-transcription conflicts has been nicely addressed and is now part of a set of data covering also other possibilities raised by the other reviewers.

We thank the reviewer for this analysis.

3. My third question was about showing the actual amounts of CDC45 protein on chromatin, rather than total levels. This becomes a key point considering the orientation that the authors give to the discussion (and the new title). A new experiment in Figure 8A addresses the question, but this could have been completed with a few additional western blots directed to other helicase components such as MCM2-7 and/or GINS proteins. The conclusion that higher amounts of helicase components on chromatin cause the roadblocks could be made stronger with a few immunoblots of little technical complexity (at least the MCM proteins are very abundant and antibodies can be obtained from many laboratories or commercial sources). It is of course possible

that only CDC45 is responsible for the effect, but the experiment in Figure EV5 with downregulation of MCM2 (no immunoblot shown) suggests otherwise.

We have included a western blot of MCM2 as requested, and accordingly modified the text.

Referee #3:

I read with interest the revised version of the work and I appreciated the impressive amount of new data added by the authors.

I carefully checked the answers provided to my comments and to those raised by the other reviewers and I think that the additional data as well as the authors' explanations are satisfactory.

In principle, I would recommend the work for publication, however, I have still some concerns with few data and some minor comments I hope the authors can deal with.

1. I thank the authors very much for the detailed explanation on their preference about representation of statistical significance marking on graphs. I still would suggest to opt for the other way but I respect the choice of the authors;

We have addressed all the queries raised by the journal regarding the statistical analysis used in each figure. We thank the reviewer for the opportunity to use the type of analysis we chose.

2. Concerning the nature of "roadblock", I would suggest to tone down the claim about the presence of reversed forks as there is not formal evaluation of them. In addition, I see that pre-treatment with Olaparib increases the slow-replicating phenotype (Fig. EV2E). One could argue that also formation of reversed forks contributes to replication slow-down and not just that reversed forks are formed upon CHK1 loss. That is, reversed forks are the barriers. Indeed, formation of reversed forks slow-down replication. The authors should include this possibility in their discussion, at least;

We have toned down the assumption that reversed forks are formed (see results) and we have discussed the possibility that reversed forks contribute to replication slow down (see discussion).

3. As far as the role of MUS81 goes, I welcome the possible explanation of the authors about how MUS81-dependent DSBs can form upon CHK1 loss or inhibition. However, as the authors added the experiment with MUS81 RNAi in Fig. EV2, why do not mention the panel? Indeed, only panel EV2F-G are mentioned in the text. In addition, I think it would be fair to mention the works reporting MUS81-dependent DSBs in CHK1-deficient cells;

We have mentioned the panel in the text (see results) and we have cited Murfuni et al, 2013 and Forment et al., 2011 (see discussion).

4. An important point concerns the sub-optimal performance of the siCHK1 batch used in the experiments shown in Fig. 2D. I'm sorry but if they know that the result may be different because of the batch variability it would be better to repeat the data using more efficient reagents or to

move the data as a supplementary material adding a panel with the control of CHK1 depletion so that readers can appreciate that.

We have included the western blot requested by reviewer 3.

Accepted 19th June 2019

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Vanesa Gottifredi
Journal Submitted to: EMBO Journal
Manuscript Number: 2018-101284

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 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.

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?	Sample size was determined based on our previous experience to ensure reproducibility. The sample size that we use are in agreement with the field´s best practices.
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?	No data was excluded, unless problems with cell culture were evident (e.g. contamination with micoplasm).
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.	For many of the DNA fiber experiments, microscopy images were taken by a "blind" investigator. For other assays, there was no blinding.
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, the information can be found in the Materials and Methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, to test normal distributions the "Column Statistics" analysis in GraphPad Prism was performed
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	Chk1 (Santa Cruz Biotechnology, G-4), Polh (Santa Cruz Biotechnology, H-300), PCNA (Santa Cruz Biotechnology, PC10), γH2AX (Millipore, 05-636), phosphoS296-Chk1 (Cell Signaling, 2349), fibrillarin (Sigma, SAB4300633), GFP (Santa Cruz Biotechnology, B-2), H2B (Santa Cruz Biotechnology, E-6), WEE1 (Santa Cruz Biotechnology, B-11), CDC7 (Santa Cruz Biotechnology, SPM171), CDC45 (Santa Cruz Biotechnology, H300), MCM2 (Abcam, ab4461), MUS81 (Santa Cruz Biotechnology, B-12), Rbp1 NTD (Cell Signaling, 14958), Ku80 (Abcam, ab236277), actin (Sigma, A2066), BdrU (GE, RPN20AB)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	U2OS are from ATCC and HCT116 are from Vogelstein lab in Baltimore. Cells were regularly tested for micoplasm contamination.

* 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 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).	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. 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

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.	No