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Human CST complex protects stalled replication forks by directly blocking MRE11 degradation of nascent-strand DNA

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1st Editorial Decision 22nd Nov 2019

Thank you for submitting your manuscript on CST roles in replication fork protection and MRE11 inhibition to our editorial office. We have now received reports from three expert referees, copied below for your information. All referees consider your findings potentially interesting, and would therefore in principle support publication as long as you should be able to decisively substantiate the key conclusions. In my view, the most important points in this respect would be:

- Clear demonstration that endogenous CST can be found at replication forks (ref 1 pt. 1)
- Follow-up on the relative roles and epistasis between CST and BRCA2 (ref 1pt. 4, ref 2 pt. 7, ref 3 pt. 4), and CST and RAD51 (ref 1 pt. 2, ref 2 pt. 8, ref 3 pt. 2
- Stronger evidence for reproducibility and statistical & biological significance of effects, as well as quantifications and controls (e.g. ref 1 pt. 3 + minor points, ref 2 pts. 2 & 3 + minor points, ref 3 pts. 1, 5, 7 + minor points)
- Improved biochemical data on nuclease inhibition by CST (ref 2 pt. 6, ref 3 pt. 6)

In addition, a number of more specific points raised in all three reports would need to be adequately addressed; on the other hand, we would consider some other, further-reaching points (e.g. ref 2 pt. 9, chromosome aberration assays asked by referee 3) beyond the scope of this revision.

Thus, should you be able to satisfactorily address the above-listed essential issues and provide the additional evidence/insights unanimously requested by all three reviewers, we would be happy to consider a revised manuscript further for EMBO Journal publication. I realize that this may require considerable further time and effort, and would therefore be open to discussing a possible extension of the default three-months revision deadline, during which publication of any competing/related work would as usual not have a negative impact on our final assessment of your own study. Please be reminded that our policy to allow only a single round of major revision will make it important to comprehensively answer to all points raised at this stage. I would further encourage you to contact me already during the early stages of revision to discuss any proposals for addressing the reviewers' concerns

Further information on preparing and uploading revised manuscript files can be found below and in our Guide to Authors.

Thank you again for the opportunity to consider this work for The EMBO Journal. I look forward to hearing from you in due time.

Referee #1:

In this study, the authors focus on the role of human CST in fork protection following genome-wide replication stalling. Previous work suggest that CST may aid in fork restart or rescue but the precise role(s) of CST in these processes is still unclear. To address this, the authors use a variety of assays to understand whether CST aids in fork protection. Their results suggest that CST can localize to stalled replication forks and block MRE11 degradation of reversed forks in a manner independent of BRCA2. Such findings would greatly enhance our understanding of the varied non-telomere roles of CST and identify a novel factor in fork protection. However, several pieces of data are not entirely convincing and additional experiments would be needed to justify their main conclusions.

Major concerns:
1. The result that CST localizes to stalled replication forks, and even active replication forks, is key to the authors' conclusions and would indicate a direct versus indirect effect on fork protection. This point is particularly important because the authors propose that CST blocks MRE11 fork degradation. Yet, previous studies did not detect CST at stalled or active replisomes (Miyaki et al. 2009. Mol Cell; Dungrawala et al. 2015. Mol Cell). Based on the data shown, I am concerned that the observed localization of CST to stalled forks may be caused by the overexpression of CST subunits. Blots demonstrating the levels of Myc-STN1 or Myc-CTC1 are not presented and it is unclear why the authors have not test endogenous CST subunits by the SIRF assay. Demonstrating that endogenous STN1 or CTC1 localization is needed to truly conclude that CST localizes to stalled and active replication forks, particularly based on conflicting results from other studies. Localization of endogenous CST by SIRF and with another replication factor by PLA in multiple cell lines would improve confidence of this key result.

2. The role of RAD51 in this process is missing from the manuscript. The authors previously showed that CST depletion led to decreased RAD51 foci formation (Chastain et al. 2016. Cell Rep) and RAD51 plays a key role in fork reversal. What happens when RAD51 is depleted along with CST? How does this affect fork protection? Does Δ700N-CTC1 affect RAD51 foci formation? If so, how does this affect fork reversal? They attempted to show that Δ700N-CTC1 still interacts with RAD51 but their data is not very convincing. The interaction between CTC1 with RAD51 shown in Figure 4D is not apparent or very weak. A negative control (vector only) is not included and CTC1 bands are in both +/- IP:Flag samples. Moreover, in Figure 6E, the authors show that CTC1 co-localizes with RPA pS33, which suggest that CST must compete with RPA for binding to reversed forks. Assessing the role of RAD51 and discussion of how these results fit into their model is needed.

3. The authors present only one representative experiment in most of the figures, which makes it hard to assess the reproducibility of their results. This is particularly important because changes in the SIRF and DNA combing experiments are not large. The authors should present multiple independent, biological replicates for these experiments, along with the error between replicates. Addback of STN1 is also needed to rule out any off-targets effects of the shRNA in the DNA combing experiments. This is done for the CTC1 knockdown, however, a western blot showing addback of WT or Δ700N CTC1 is not shown (Figure S2 and 4E).

4. The argument for synthetic lethality, when STN1 and BRCA2 are co-depleted, requires more supporting data. The phenotypes measured appear to be additive but no data is provided on cell

viability or cell cycle changes when both STN1 and BRCA2 are depleted. The authors mention that DNA fiber was not able to be performed due to low cells numbers but data for this is not shown.

Minor concerns:
1. The introduction does not mention other studies demonstrating non-telomeric roles of CST, such as in DSB repair, RAD51 recruitment to GC-rich DNA, dormant origin activation and origin licensing. These studies are important to understanding the context and possible interpretations of the current study.

2. The authors should provide the number of cells/fibers that were analyzed for each sample and the mean intensities for the dot plots.

3. Throughout the paper, the incubation periods with HU vary, which could affect interpretation of the results. For example, HU is used at 4 mM for 3 h in Figure 1 but 2 mM for 6 h in Figure 2B and 3 h in Figure 2C. This could lead to changes in the association of replication factors at stalled forks and whether the fork is stalled or collapsed (Dungrawala et al. 2015. Mol Cell). Is there a reason for the different incubation times?

4. Page 3, paragraph 2: remove "s" from protects "...of mechanisms protects genome stability..."

5. Figure 1C: -EdU controls should be included.

6. Figure 2A: TEN1 knockdown levels are not shown.

7. The interpretation that levels of ssDNA are increased with STN1 depletion and remain longer in STN1 depleted cells does not seem consistent with the presented data. Relative RPA foci start at different levels in the graph (time=0) so the changes in the recovery rate, when accounting for the increase, seem to be very minor, or non-existent. A similar experiment was also previously done in HeLa cells and showed no changes in RPA levels after release from HU treatment (Stewart, et al. 2012. EMBO J). These conflicting results should be addressed as well as a clearer representation of the data.

8. Representative images of a field of DNA fibers should be included in the supplementary data to demonstrate the staining quality.

9. The purpose of Figure 3G is unclear. Treatment with CPT and MMS seems irrelevant to study, as all other experiments are done with HU and treatment with these compounds can induce defects in different pathways (i.e. are not restricted to MRE11-dependent fork degradation). The authors should either clarify why these have been included or remove them from the study.

10. Figure 4B: An empty vector control should be included.

11. Figure 5D: The CST-DNA complexes appear to be stuck in the wells. Could this be caused by aggregation of the WT CST? Quantitative analysis of the binding constants (Kd) would be helpful to observe true differences between the WT and Δ700N CST.

12. Figure S4: No reference is given for where this data comes from.

13. Figure S5: What do the different colors represent?

Referee #2:

In this manuscript, Lyu et al. have nicely extended/complemented previous findings about the genome-wide function of the CST complex in replication stress management, besides its corroborated involvement in telomere maintenance. In particular, they show that the CST complex is recruited to stalled forks and protect them from MRE11-mediated nucleolytic degradation. In light of previously reported molecular functions and interacting partners, the CST complex could promote replication fork stability by at least two independent mechanisms: a) recruiting RAD51 (as for BRCA2-mediated fork protection; Chastain et al. 2016) or b) promoting pol-alpha dependent DNA synthesis (as shown for DSB repair, Mirman et al. 2018, Barazas et al. 2018). Surprisingly, the authors provide biochemical evidence that the CST complex directly blocks in vitro MRE11 nuclease activity on a 5'-overhang substrate mimicking the extruded arm of a reversed fork, by mean of its DNA binding activity. Additional cell-biology readouts in the manuscript - which would need strengthening - are consistent with this interpretation.

The authors also propose that the CST complex has a non-redundant function with BRCA2 in promoting genomic stability under endogenous replication stress, localizing to a different set of stalled forks. Although this aspect of the manuscript is not fully developed, the authors speculate that the CST complex may act at G-rich regions experiencing replication stress, where BRCA2 cannot bind. It is indeed an intriguing hypothesis that cells have evolved two different protection systems (RAD51-dependent and -independent) to cope with replication stress at different regions, possibly to prevent RAD51-pathway at repetitive sequence, where it could lead to detrimental recombination outcomes. This would be consistent with the milder effect of CST in nascent strand protection compared to BRCA2, at least as reported in the literature.

In my view, this manuscript has potential to be further considered for publication in EMBO journal. However, several key conclusions would strictly need a significantly extended experiments, additional controls and refined analyses.

MAJOR POINTS:

1. Two important predictions of the proposed model are that: a) ssDNA formation in absence of the CST complex under replication stress (Fig. 2) is dependent on unscheduled fork degradation carried out by MRE11 and b) CST-depleted cells display an increased MRE11 recruitment at HU-stalled forks by SIRF (Fig. 1B). It seems that the authors have the experimental set-up to test these important predictions, which would allow to confirm or refine their proposed model.

2. The experiments in Fig. 2C do not provide information on fork restart, but rather on residual ssDNA accumulation after HU removal. The residual accumulated ssDNA upon CST inactivation may reflect different intermediates than forks delaying restart (e.g. inaccurate repriming at restarting forks). As the authors can obviously perform DNA fiber assays, fork restart should be assessed by a proper labelling protocol, where the second label follows an HU arrest. This will allow testing whether efficiency (% red only) and/or velocity (green track length) of fork restart are affected upon CST inactivation.

3. It would be essential to include BRCA2-defective cells as control for fork degradation phenotype. The impression is that fork degradation is quite mild upon CST inactivation (adding the value of the median in Fig. 3B-F would help), when compared to similar previously described defects (BRCA2, FA proteins). Statistical significance among fiber data sets may be misleading, as the high number of

data points may lead to highly significant differences, even for very mild effects (as I think they have in this case).

4. It is not really clear why the authors have used CPT and MMS for clonogenic experiments, if HU has been used throughout the study. Moreover, the same group has previously shown that CTC1-depleted cells are sensitive and display chromosomal abnormalities in response to HU. It would be essential to test whether HU sensitivity of CST-defective cells is MRE11-dependent and BRCA2-additive, which may again support or refine the proposed model.

5. The involvement of pol-alpha as gap-filling mechanism of CST-mediated fork protection could be easily tested by commercially available pol-alpha inhibitors.

6. The biochemical inhibition of MRE11 nuclease activity in vitro by the CST complex (Fig. 5) is novel and probably the most interesting evidence in this manuscript. Hence, these data should be complemented with more control experiments, to make this observation more solid. In particular it would be important to assess:
a. if CST really blocks MRE11 access to ss/ds junction by using a substrate with biotin-streptavidin terminal blocks;
b. if this CST inhibitory effect is specific for MRE11 or is general for any 3'-5' exonuclease (for example bacterial ExoIII);
c. the effect of RPA in this reaction;
d. if this inhibition is sequence specific; for this purpose it would be interesting to test the same effect on a G- or C-rich (on the ssDNA part) substrate.

7. The authors claim they could not perform DNA fiber experiments in BRCA2- and CST-double-depleted cells because of the low number of replicating cells (even though Fig 6D shows that just half of the cell population does not incorporate BrdU in double knockdown compared to control). It seems to this referee that this is an essential experiment for the conclusions of this paper and that experimental conditions could/should be found to perform it. It is likely that the long-term consequences of protein depletion are attenuated at early time points after siRNA-mediated knockdown. Several groups have previously shown fork degradation phenotypes at relatively short times after transient BRCA2 depletion, when cell cycle effects are not yet observed. The authors should identify proper conditions to perform these key experiments, as the data currently present in Fig. 6 do not seem to sufficiently support the strong conclusions on epistasis included in the manuscript.

8. The authors have previously shown that the CST complex interacts with and recruits RAD51 to stalled forks. Thus, despite their biochemical data, one could hypothesise that CST promotes fork stability indirectly via RAD51 stabilization on reversed forks and not by directly protecting them from MRE11-dependent nucleolytic digestion. The authors should directly assess RAD51 recruitment to stalled forks by SIRF and explicitly discuss this hypothesis based on these new results. This point may also help clarifying the epistatic relationship with BRCA2 in fork protection.

9. As CST foci largely overlap with telomeres (Fig 4B) even upon HU treatment, it would be important to discriminate more specifically what structural determinants differently recruit CST or BRCA2 to stalled forks. Do BRCA2 and CST differentially localise at G4-forming (not only telomeric) regions experiencing replicative stress? While I realise that this aspect may be beyond the scope of this manuscript, the authors propose here an intriguing hypothesis. They should either remove it from the manuscript, or obtain at least some initial evidence to support it.

MINOR POINTS:

1. Fig1A is unnecessary (at least as a main figure) and 1B could be easily incorporated in Fig 2.

2. The panels 4A-C are controls for the key experiment in this figure (4D) and should be probably moved to the Supplementary figures. It is confusing for the flow of the manuscript to assess telomere recruitment in the main figure. In fact, it would be much more relevant to test directly recruitment to stalled forks of the delta700N mutant, which may nicely complement Fig. 4D in the main figure.

3. Please add nucleotide markers in Fig 5 (DNA gels).

4. BRCA2 downregulation efficiency should be shown by Western Blot.

5. A more absolute quantification of yH2AX staining should be used for Fig. 6C, using number of foci or mean intensity, assessing statistically significant differences between single and double knockout.

6. A quantitative measurement of colocalization should be done for the data in Fig. 6E.

7. A colour-code legend should be included in Figure S5 to discriminate amplifications, deletions, mutation etc.

8. Additional experimental details should be generally added to figure legends and/or Methods (e.g. statistical analysis in Fig. 5, timing of HU treatment in Fig 6E, antibodies used for SIRF, antibody dilutions used for the different experiments, …).

Referee #3:

This study by Lyu et al proposes a new function for the CST complex in preventing excessive nascent strand degradation at stalled replication forks, indicating an important role in maintain genomic instability. Using a variety of experimental approaches, this study showed that CST localizes to stalled replication forks, therefore preventing persistent ssDNA accumulation and nascent strand degradation caused by MRE11 nuclease. Using DNA fiber analysis and biochemical systems, the author further imply CST binding and protection of reversed structures. Importantly, there is a spatial separation of CST with BRCA2, a major fork protection protein, implying alternative pathways.

Overall, the study is well designed and will undoubtedly add understanding to the replication genome stability field. The authors should however consider the following points for their revision.

1• The authors propose that the data in Fig 2C indicates a delay in replication recovery when CST is depleted. These data is weak, and RPA32 reduction could have several reasons. A more informative assay for replication recovery would be DNA fiber analysis.
2• It is not clear how CST protects from degradation. The authors mention a direct interaction with RAD51, the known actor for fork protection downstream of BRCA2. Is CST required for RAD51 foci

formation or retention (stabilization)?
3• The Brca2 antibody use for IF studies needs to be validated for specificity.
4• The spatial separation of BRCA2 and CST is intriguing: is there a temporal or solely a spatial separation? E.g. are there more BRCA2 foci in early replicating cells compared to more CST foci in late replicating cells (consistent with CST's function at more difficult to replicate regions such as telomers, which typically replicate late in S-phase). It also would be informative to show alternative methods.
5• SIRF assays need to be normalized to total Edu content, either by co-click with Alexa 488 or at a minimum by normalization slides with EdU-EdU PLA. It is likely that EdU concentrations increase with continued EdU exposure, and decrease with HU if it is degraded, and this will skew the results.
6• The biochemical assays require a negative control to show that the nuclease inhibition by CST is specific and not simple substrate titration availability. E.g. does SSB or RPA also block degradation in the system?
7• Quantification of Fig 4B and 6E would be helpful.

- minor points
• The extent of fork protection seems only very mild- controls with BRCA2 knock down for direct comparison would be informative
• LUC abbreviation is not explained in text
• Fig3 E and 3F: include statistics to compare group1 (con) and group 3 con+SMARCAL/ZRANB3

- at author's discretion:

CST depletion only mildly sensitizes cells to fork stalling agents and causes only a minor increase in micronuclei formation. Metaphase chromosome aberration assay may be more informative

Point-by-point response:

Dear Reviewers,

We are grateful to you for your insightful and constructive comments that have helped us improve the original submission. As you know, in the middle of our revision, the COVID-19 outbreak forced many universities across the US to shut down their research labs. We were no exception. Our building was completely shut down for nearly 3 months, and then cautiously reopened with tight restrictions in order to comply with social distancing laws. The lockdown caused significant disruption and delay, making it difficult to obtain data in a timely manner. Nonetheless, our lab gave this revision the highest priority after lab reopen. While our access to common equipment and core facilities was greatly limited due to social distancing, we have made our best efforts to address your concerns, and hope you will find the revised version much improved. We greatly appreciate your patience!

Major changes in the main text are labeled with vertical lines on the right side of the margin.

Referee #1:

In this study, the authors focus on the role of human CST in fork protection following genome-wide replication stalling. Previous work suggest that CST may aid in fork restart or rescue but the precise role(s) of CST in these processes is still unclear. To address this, the authors use a variety of assays to understand whether CST aids in fork protection. Their results suggest that CST can localize to stalled replication forks and block MRE11 degradation of reversed forks in a manner independent of BRCA2. Such findings would greatly enhance our understanding of the varied non-telomere roles of CST and identify a novel factor in fork protection. However, several pieces of data are not entirely convincing and additional experiments would be needed to justify their main conclusions.

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> There are two questions here: whether CST localizes at stalled forks, and whether it is at active forks. We have now used SIRF to detect the localization of endogenous CTC1 and

STN1 at stalled forks in two different cell lines U2OS and HCT116. We have replaced the original Figure 1 with new results (revised Figure 1), which show that endogenous CTC1 and STN1 localize at stalled forks. This finding is also consistent with our repeated observation that CST colocalizes with RAD51 foci in response to HU treatment, which we have published previously (Chastain et al, Cell Rep 2016, Wang et al, NAR 2018).

After the initial submission of our manuscript, a very recent paper "RIF1 promotes replication fork protection and efficient restart to maintain genome stability" Nature Comm. 2019 reports that STN1 (aka OBFC1) can be detected at stalled forks via iPOND-MS (data in Supplemental Table 1 in this paper), consistent with our SIRF finding. We think one possible reason that CST is difficult to detect by iPOND might be its very low abundance.

Our SIRF also detected CST localization at active forks (revised Figure 1), which is consistent with the recent report (Yilin, et al., Life Science Alliance, 2019) showing that CST directly interacts with the MCM complex during the normal replication. We also notice that the SIRF foci number of CST at active forks is fewer compared to that at stalled replication forks.

2. The role of RAD51 in this process is missing from the manuscript. The authors previously showed that CST depletion led to decreased RAD51 foci formation (Chastain et al. 2016. Cell Rep) and RAD51 plays a key role in fork reversal. What happens when RAD51 is depleted along with CST? How does this affect fork protection? Does Δ700N-CTC1 affect RAD51 foci formation? If so, how does this affect fork reversal? They attempted to show that Δ700N-CTC1 still interacts with RAD51 but their data is not very convincing. The interaction between CTC1 with RAD51 shown in Figure 4D is not apparent or very weak. A negative control (vector only) is not included and CTC1 bands are in both +/- IP:Flag samples. Moreover, in Figure 6E, the authors show that CTC1 co-localizes with RPA pS33, which suggest that CST must compete with RPA for binding to reversed forks. Assessing the role of RAD51 and discussion of how these results fit into their model is needed.

This comment contains three major points: 1) the relationship between CST and RAD51 at stalled forks; 2) how does Δ700N affect RAD51 foci formation; and 3) whether CST competes with RPA for binding to reversed forks. We are extremely interested in understanding the functional relationship between CST/RAD51/RPA. We would like to address the third question first, and then address the more complicated CST/RAD51 relationship question.

1) Whether CST competes with RPA for binding to reversed forks: we have purified and tested the reversal fork binding ability of CST and RPA to ds-ssDNA side-by-side. Our results show that CST and RPA have a similar binding affinity to ss/ds junctions. However, unlike CST, RPA lacks the function of reversal forks protection from MRE11 (see new Supplemental Figure S4). Please see our more detailed response to Reviewer 2 Major point 6c). In addition, our preliminary data also show that CST does not compete with RPA for binding to the ds-ssDNA substrate by the competing experiment (unpublished). Thus the relationship between CST and RPA in cells is more complexed than expected. This is an interesting topic that we are actively pursuing now.

2) The relationship between CST and RAD51 at stalled forks: RAD51 is essential for both mediating fork reversal and protecting reversed forks from degradation. In order to study the

functional relationship of CST and RAD51, we performed RAD51 SIRF analysis and found that the binding of RAD51 to the stalled forks diminished in STN1 depleted cells (new Figure 7A). This result is consistent with our previous results showing that CST depletion attenuates RAD51 foci formation and RAD51 recruitment to GC-rich sites after fork stalling (Chastain et al, Cell Rep 2016; Wang, et al NAR 2018). In addition, we co-depleted of CST and RAD51 as the reviewer suggested, and found that depletion of RAD51 abolishes nascent strand degradation in CST deficient cells, indicating that the degradation relies on RAD51-mediated fork reversal. New results are included in the new Figure 7.

We have observed that CST deficiency only partially decreases RAD51 binding to DNA (Chastain et al. 2016. Cell Rep). Similarly, we now observe that the decrease of RAD51 SIRF signal in STN1 depleted cells is also partial (new Figure 7A). These findings indicate that RAD51 can be recruited to stalled forks in a CST-independent manner. We would like to clarify that in this manuscript, we do not exclude the possibility that CST may also recruit RAD51 to stalled forks to protect fork stability. The focus of this manuscript is to reveal a new mechanism that CST can bind to stalled forks and directly block MRE11 degradation. The two protection mechanisms may co-exist. We have discussed the role of RAD51 in Discussion and modified our model (see the new Figure 7D).

3) How does ∆700N affect RAD51 foci formation: We did RAD51 IF staining in Δ700N-CTC1 cells and results showed that this mutant cannot rescue the defect of RAD51 foci formation in knockdown cells. This data is now provided in the new Figure 7B.

4) ∆700N interaction with RAD51: We have redone the ∆700N/RAD51 co-IP experiment with vector only control more than three times. Each time we observe ∆700N interacts with RAD51. To better we have replaced the original co-IP blot with a new one in Figure 5C.

3. The authors present only one representative experiment in most of the figures, which makes it hard to assess the reproducibility of their results. This is particularly important because changes in the SIRF and DNA combing experiments are not large. The authors should present multiple independent, biological replicates for these experiments, along with the error between replicates. Addback of STN1 is also needed to rule out any off-targets effects of the shRNA in the DNA combing experiments. This is done for the CTC1 knockdown, however, a western blot showing addback of WT or Δ700N CTC1 is not shown (Figure S2 and 4E).

When we perform DNA fiber analysis and SIRF, we routinely do multiple independent biological replicates (from culturing cells, CldU/IdU labeling to DNA fiber assay and then to acquiring images and analysis) to ensure reproducibility. Per reviewer's request, we have included data from biological replicates in supplemental figures.

We have performed the rescue experiment by expressing RNAi-resistant STN1 and the data are included in the revised Figure 2D. Western blot is also provided in the same figure. Our result shows that reintroducing STN1 in knockdown cells fully rescued degradation, thus excluding the off-target effects.

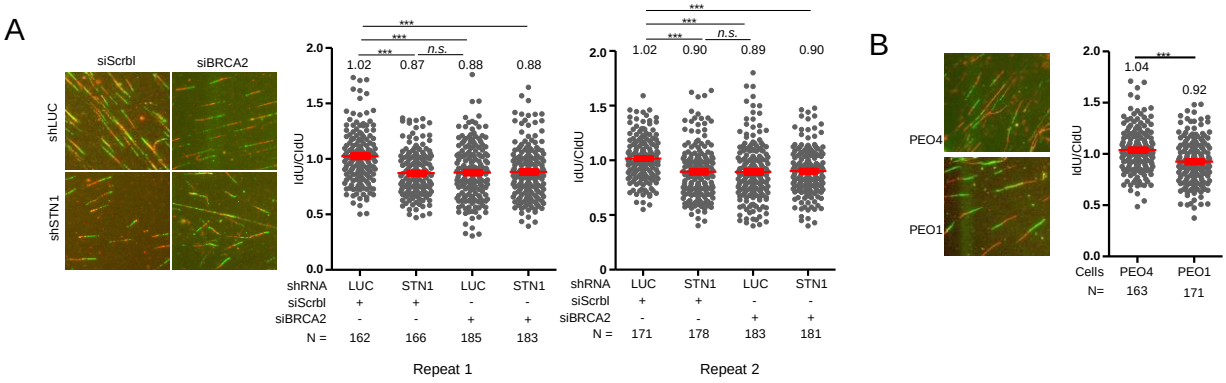
We have included CTC1 knockdown and addback WT or ∆700N CTC1 western blot in Supplemental Figure S5A.

4. The argument for synthetic lethality, when STN1 and BRCA2 are co-depleted, requires more

supporting data. The phenotypes measured appear to be additive but no data is provided on cell viability or cell cycle changes when both STN1 and BRCA2 are depleted. The authors mention that DNA fiber was not able to be performed due to low cells numbers but data for this is not shown.

This question is similar to Reviewer 2, major point 7, so we are addressing this concern for both reviewers here.

In order to know whether co-depletion of CST and BRCA2 has additive effect on nascent strand degradation, we have tried DNA fiber analysis in STN1/BRCA2 double knockdown cells as suggested by this reviewer, along with STN1 and BRCA2 single knockdowns side-by-side. However, STN1/BRCA2 co-depletion induces massive cell detachment from dish (new Supplemental Figure S6C). These detached cells were removed from analysis by washing steps in the DNA fiber assay (to remove CldU/IdU labeling before adding HU). When we checked BRCA2 and STN1 knockdown in both the attached and detached cells, we found that attached cells had recovered STN1 expression (new Supplemental Figure S6C). Not surprisingly, when we performed DNA fiber analysis using attached cells, each time we failed to detect additive effect on fiber degradation in double knockdown cells (see data below). Since the DNA fiber analysis requires cell attachment, it is not feasible to obtain true nascent strand degradation data from co-depleted cells.



(A) Nascent strand DNA degradation in cells that remain attached to the dish after STN1 and BRCA2 co-depletion. ***P<0.001. (B) Nascent strand DNA degradation in PEO1 and PEO4. ***P<0.001.

In contrast, assays for detecting other genome instability markers (micronuclei, anaphase bridge, gH2AX) do not involve washing and we collected all cells for analyses. Therefore, we were able to detect additive effects of double knockdown in these assays.

Under our experimental condition, we observed relatively mild fiber shortening in both siBRCA2 U2OS cells and the BRCA2-deficient cell line PEO1 (see figure above). We notice that different labs have reported various levels of nascent strand degradation in BRCA2 knockdown cells, ranging from mild shortening to 40% shortening (16-22% in Anika, Molecular Cancer Therapeutics, 2018; 20-31.4% in Sofija, et.al, Nature Communications, 2017; 25% in Bartlomiej, et.al, iScience, 2019; 20-30% in Huzefa, et al, Molecular Cell,2017; 30-40% in Angelo et.al, Molecular Cell, 2017). The reason underlying such discrepancies is unclear.

We have examined whether CST-defective cells are more sensitive to HU treatment and are BRCA2-additive by using chromosomal abnormality assay (revised Figure 6E). Result shows that CST and BRCA2 double knockdown further increases genome instability under HU treatment, further supporting the synthetic lethality relationship of CST and BRCA2. We have included the new data in the revised Figure 6E.

Minor concerns:

1. The introduction does not mention other studies demonstrating non-telomeric roles of CST, such as in DSB repair, RAD51 recruitment to GC-rich DNA, dormant origin activation and origin licensing. These studies are important to understanding the context and possible interpretations of the current study.

We have included a paragraph describing non-telomeric non-replication stress roles of CST in “Introduction” on Page 5.

2. The authors should provide the number of cells/fibers that were analyzed for each sample and the mean intensities for the dot plots.

We have included the number of cells/fibers in all figures or figure legends.

3. Throughout the paper, the incubation periods with HU vary, which could affect interpretation of the results. For example, HU is used at 4 mM for 3 h in Figure 1 but 2 mM for 6 h in Figure 2B and 3 h in Figure 2C. This could lead to changes in the association of replication factors at stalled forks and whether the fork is stalled or collapsed (Dungrawala et al. 2015. Mol Cell). Is there a reason for the different incubation times?

We have redone experiments in Figure 2B by using HU 2 mM for 3 hr. All experiments presented in our revised manuscript are using 3hr HU treatment.

Both 2 mM and 4 mM HU are commonly used in published papers for inducing fork stalling (Su et.al, Nature communications, 2019) (Eva et.al., Mol Cell, 2010) (Giuseppe, et.al, EMBO J, 2016) (Andrea, et.al, JCB,2019). At the early stages of our study, we performed IF assays using 2 mM HU. Later when we started SIRF assay, we noticed that 4 mM HU is more widely used in published SIRF studies (Martin et.al, Mol Cell,2018) (Chirantani et. al, Nature communications, 2019) (Jadwiga et.al, Mol Cell 2019), and thus we used 4 mM in our fiber assays. In our experimental settings, we do not observe significant difference between 2 mM and 4 mM HU treatment.

4. Page 3, paragraph 2: remove "s" from protects "...of mechanisms protects genome stability..."

Corrected.

5. Figure 1C: -EdU controls should be included.

We have included EdU controls in Supplemental Figure S1B. Very few SIRF positive cells were observed without EdU, suggesting that SIRF assay was specific for determining protein localization at replication forks.

6. Figure 2A: TEN1 knockdown levels are not shown.

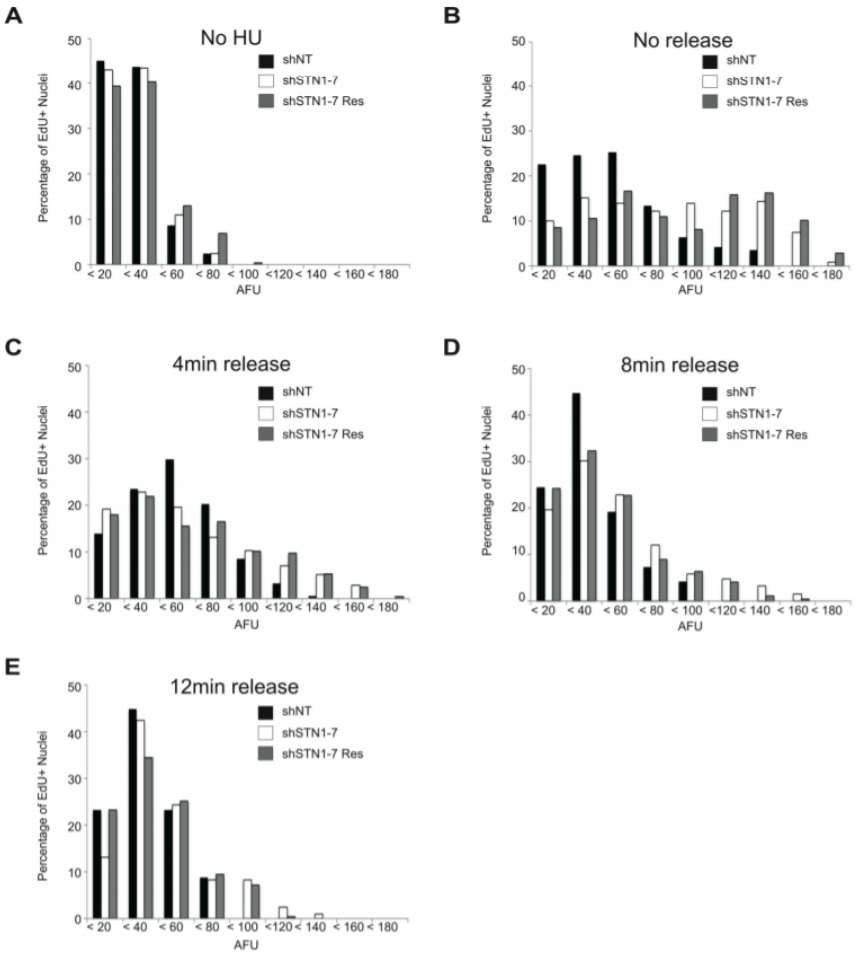
To our knowledge there is no commercially available TEN1 antibody. In the revised manuscript, we have replaced the RPA staining data with BrdU IF to show the increase of ssDNA in STN1 depleted cells (Figure 2B). As a result, the TEN1 knockdown data has been removed.

7. The interpretation that levels of ssDNA are increased with STN1 depletion and remain longer in STN1 depleted cells does not seem consistent with the presented data. Relative RPA foci start at different levels in the graph (time=0) so the changes in the recovery rate, when accounting for the increase, seem to be very minor, or non-existent. A similar experiment was also previously done in HeLa cells and showed no changes in RPA levels after release from HU treatment (Stewart, et al. 2012. EMBO J). These conflicting results should be addressed as well as a clearer representation of the data.

Our revised manuscript has expanded significantly from the original submission. Since fork restart is a physiological process separate from the fork stability maintenance, we think that including fork restart will distract the focus of the manuscript. Thus, we have removed the fork recovery data from the revised manuscript and focus on fork stability.

Nevertheless, we would like to inform the reviewer that our RPA IF data were independently collected by two individuals in our lab, and we are very confident in our data. We are well aware of the data published in Stewart et al 2012 EMBO J and their claim that STN1 knockdown does not change RPA levels after release from HU. However, after carefully looking at their data, we think their result is largely consistent with ours, despite the authors' claim. Below is our analysis of their published data.

Stewart et al provided the quantification of their RPA staining data in Supplemental Figure 8, which is copied below. In panel A, which shows the "no HU" result, the majority of RPA fluorescence intensity in the shNT control sample is <80 AFU, suggesting that RPA32 signal with < 80 AFU is the baseline RPA staining during normal replication. In panel B, which shows RPA fluorescence intensity under HU treatment, we notice that RPA baseline signal (<80 AFU) does not change in the shSTN1 sample (presumably because this is baseline signal). However, >80 AFU RPA staining in shSTN1-7 is much stronger than shNT. We think >80 AFU signals are real RPA staining induced by HU. This is the same as our data (time 0 after HU treatment). (It's also interesting to notice that their rescue experiment did not rescue for reasons unknown to us.) In panels C,D,E, which show RPA fluorescence intensity after release from HU at 4, 8, 12 min, the >80 AFU signals in shSTN1-7 are stronger than those in shNT. In fact, at 12 min after release, shSTN1-7 sample still has considerable amount of >80 AFU signals while the control shows undetectable >80 AFU signal, indicating that STN1 knockdown delays the recovery of RPA staining after HU release. In summary, we believe the published results in Stewart et al are largely consistent with our results that were included in our original manuscript, i.e. ssDNA are increased with STN1 depletion after HU treatment and STN1 depleted cells need longer time to reduce ssDNA amount after HU release.



8. Representative images of a field of DNA fibers should be included in the supplementary data to demonstrate the staining quality.

They are now included in all Supplemental Figures containing DNA fiber results.

9. The purpose of Figure 3G is unclear. Treatment with CPT and MMS seems irrelevant to study, as all other experiments are done with HU and treatment with these compounds can induce defects in different pathways (i.e. are not restricted to MRE11-dependent fork degradation). The authors should either clarify why these have been included or remove them from the study.

We agree. This has been removed from the revised manuscript.

10. Figure 4B: An empty vector control should be included.

Thank you for pointing it out. We have included the vector control.

11. Figure 5D: The CST-DNA complexes appear to be stuck in the wells. Could this be caused by

aggregation of the WT CST? Quantitative analysis of the binding constants (Kd) would be helpful to observe true differences between the WT and Δ700N CST.

We had replaced a new EMSA of the CST-DNA complexes performed in 0.8% agarose gel and most of them can run into the wells, suggesting the CST-DNA complexes may just form a large complex but not caused by aggregation (Fig. 4D). Additionally, since CST proteins were purified through a size-exclusion column in the last step of the purification procedure (see Methods for the detail), any soluble aggregates of CST were excluded during purification.

Since Δ700N CST does not bind DNA in our system, we cannot determine the Kd of this truncated variant.

12. Figure S4: No reference is given for where this data comes from.

This data is from the website of Kaplan-Meier plotter (https://kmplot.com/analysis/), and the citation has been added in the revised manuscript per the website's suggestion.

13. Figure S5: What do the different colors represent?

We have updated this figure using the most recent available data in cBioPortal, and added color-code legends. The new images are Included in Supplemental Figure S8B.

Referee #2:

In this manuscript, Lyu et al. have nicely extended/complemented previous findings about the genome-wide function of the CST complex in replication stress management, besides its corroborated involvement in telomere maintenance. In particular, they show that the CST complex is recruited to stalled forks and protect them from MRE11-mediated nucleolytic degradation. In light of previously reported molecular functions and interacting partners, the CST complex could promote replication fork stability by at least two independent mechanisms: a) recruiting RAD51 (as for BRCA2-mediated fork protection; Chastain et al. 2016) or b) promoting pol-alpha dependent DNA synthesis (as shown for DSB repair, Mirman et al. 2018, Barazas et al. 2018). Surprisingly, the authors provide biochemical evidence that the CST complex directly blocks in vitro MRE11 nuclease activity on a 5'-overhang substrate mimicking the extruded arm of a reversed fork, by mean of its DNA binding activity. Additional cell-biology readouts in the manuscript - which would need strengthening - are consistent with this interpretation.

The authors also propose that the CST complex has a non-redundant function with BRCA2 in promoting genomic stability under endogenous replication stress, localizing to a different set of stalled forks. Although this aspect of the manuscript is not fully developed, the authors speculate that the CST complex may act at G-rich regions experiencing replication stress, where BRCA2 cannot bind. It is indeed an intriguing hypothesis that cells have evolved two different protection systems (RAD51-dependent and -independent) to cope with replication stress at

different regions, possibly to prevent RAD51-pathway at repetitive sequence, where it could lead to detrimental recombination outcomes. This would be consistent with the milder effect of CST in nascent strand protection compared to BRCA2, at least as reported in the literature.

In my view, this manuscript has potential to be further considered for publication in EMBO journal. However, several key conclusions would strictly need a significantly extended experiments, additional controls and refined analyses.

MAJOR POINTS:

1. Two important predictions of the proposed model are that: a) ssDNA formation in absence of the CST complex under replication stress (Fig. 2) is dependent on unscheduled fork degradation carried out by MRE11 and b) CST-depleted cells display an increased MRE11 recruitment at HU-stalled forks by SIRF (Fig. 1B). It seems that the authors have the experimental set-up to test these important predictions, which would allow to confirm or refine their proposed model.

> This is an excellent point. We have performed MRE11 SIRF in CST knockdown cells and included the new data in the new Figure 3A. Our results show that MRE11 recruitment to stalled forks is indeed increased after CST downregulation. We also measured ssDNA formation using native BrdU staining in CST knockdown cells treated with and without MRE11 inhibitor mirin, and the results shows that the increased ssDNA formation in CST knockdown cells can be rescued by mirin (new Figure 3B), consistent with our DNA fiber results that fork degradation in CST deficient cells is caused by MRE11 nuclease activity.

2. The experiments in Fig. 2C do not provide information on fork restart, but rather on residual ssDNA accumulation after HU removal. The residual accumulated ssDNA upon CST inactivation may reflect different intermediates than forks delaying restart (e.g. inaccurate repriming at restarting forks). As the authors can obviously perform DNA fiber assays, fork restart should be assessed by a proper labelling protocol, where the second label follows an HU arrest. This will allow testing whether efficiency (% red only) and/or velocity (green track length) of fork restart are affected upon CST inactivation.

> This point is similar to Reviewer 1 Minor point 7. Please see our response there. We have removed this set of data in the revised manuscript.

3. It would be essential to include BRCA2-defective cells as control for fork degradation phenotype. The impression is that fork degradation is quite mild upon CST inactivation (adding the value of the median in Fig. 3B-F would help), when compared to similar previously described defects (BRCA2, FA proteins). Statistical significance among fiber data sets may be misleading, as the high number of data points may lead to highly significant differences, even for very mild effects (as I think they have in this case).

> We performed siBRCA2 as described in published reports and did DNA fiber analysis along with STN1 knockdown side-by-side. Please see our response to Reviewer 1 Major point 4.

Statistical analyses among DNA fiber assays were done with appropriate methods: one-way ANOVA analysis with post hoc Tukey when more than two groups were assessed and Mann Whitney test when two groups were assessed, both of which are widely used, for example, the one-way anova analysis with post hoc Tukey is used in Meettu et al, NAR, 2016; Yang et al, JCB,2017; Luciana et al, Cell Death & Disease 2019, and the Mann Whitney test is used in John, et al., NAR, 2020; Joonyoung, et al., Mol Cell Biol., 2018; Sabrina, et al., eLife, 2016; Francois, et al., Cancer research, 2018.

For each assay, we performed at least 2-3 completely independent experiments (from knockdown, culturing cells, labeling, to fiber analysis). In key experiments the data were collected by two individuals independently for validation. To ease reviewer's mind on reproducibility of the data, we have included data from biological replicates for DNA fiber assays in supplemental figures.

4. It is not really clear why the authors have used CPT and MMS for clonogenic experiments, if HU has been used throughout the study. Moreover, the same group has previously shown that CTC1-depleted cells are sensitive and display chromosomal abnormalities in response to HU. It would be essential to test whether HU sensitivity of CST-defective cells is MRE11-dependent and BRCA2-additive, which may again support or refine the proposed model.

We have examined whether CST-defective cells are more sensitive to HU treatment and are BRCA2-additive by using chromosomal abnormality assay (revised Figure 6E). Result shows that CST and BRCA2 double knockdown further increases genome instability under HU treatment. We have included the new data in the revised Figure 6E.

The question of whether HU sensitivity of CST-defective cells is MRE11-dependent is similar to Major point 1. We have examined the genome stability by testing the ssDNA formation in CST deficient cells with and without mirin treatment. This new result is included in the revised Figure 3B.

We have removed CPT and MMS data to avoid confusion.

5. The involvement of pol-alpha as gap-filling mechanism of CST-mediated fork protection could be easily tested by commercially available pol-alpha inhibitors.

To study nascent strand degradation, we labelled cells with CldU/IdU and subsequently treated cells with HU to stall replication forks. HU induces fork stalling by dNTP depletion, and thus HU treatment should inhibit all DNA synthesis including pol-alpha mediated fill-in. For this reason, we do not think that the gap-filling can occur during HU treatment. It would not be feasible to test the role of gap-filling in fork protection by adding Pol-alpha inhibitor in the presence of HU.

6. The biochemical inhibition of MRE11 nuclease activity in vitro by the CST complex (Fig. 5) is novel and probably the most interesting evidence in this manuscript. Hence, these data should be complemented with more control experiments, to make this observation more solid. In particular it would be important to assess:
a. if CST really blocks MRE11 access to ss/ds junction by using a substrate with biotin-streptavidin terminal blocks;

We had examined whether CST prevents MRE11 processing by using the substrate with 5' biotin-streptavidin terminal blocks. However, we found that the biotin-streptavidin substrate somehow inhibits the enzymatic activity of human MRE11. Relevant studies from others to examine human MRE11 activity also used the substrate with phosphorothioate bonds instead of biotin-streptavidin blocks (Zadorozhny et al, 2017).

b. if this CST inhibitory effect is specific for MRE11 or is general for any 3'-5' exonuclease (for example bacterial ExoIII);

We had examined whether CST could protect the substrate from bacterial ExoIII degradation. The result shows that CST (200 nM) only slightly protects the substrate (~ 37%) from ExoIII degradation while the same concentration of CST entirely protects substrate (~ 90%) from MRE11 degradation (Compare Fig 4E (i) lane 6 and Supplementary Fig S4D lane 4).

c. the effect of RPA in this reaction;

To test whether RPA could protect the DNA substrate from MRE11 degradation, we first determined the DNA-binding activity of RPA. CST was also included for a side-by-side comparison. The result shows that both RPA and CST bind this DNA substrate vividly with a comparable DNA-binding affinity (Supplementary Figure S4B). Importantly, we found that CST (200 nM) can protect DNA (~ 90%) from MRE11 degradation; while the same concentration of RPA significantly lacks this ability (~ 15%; Supplementary Figure S4C).

In conclusion, the ExoIII and RPA experiments further indicate the specificity of CST against the MRE11-mediated DNA degradation.

d. if this inhibition is sequence specific; for this purpose it would be interesting to test the same effect on a G- or C-rich (on the ssDNA part) substrate.

To test whether the inhibition of MRE11 activity is sequence specific, we used a G-rich 5' overhang substrate and found CST has a similar inhibitory effect with this G-rich substrate (Supplementary Figure S4E).

7. The authors claim they could not perform DNA fiber experiments in BRCA2- and CST-double-depleted cells because of the low number of replicating cells (even though Fig 6D shows that just half of the cell population does not incorporate BrdU in double knockdown compared to control). It seems to this referee that this is an essential experiment for the conclusions of this paper and that experimental conditions could/should be found to perform it. It is likely that the long-term consequences of protein depletion are attenuated at early time points after siRNA-mediated knockdown. Several groups have previously shown fork degradation phenotypes at relatively short times after transient BRCA2 depletion, when cell cycle effects are not yet observed. The authors should identify proper conditions to perform these key experiments, as the data currently present in Fig. 6 do not seem to sufficiently support the strong conclusions on epistasis included in the manuscript.

This is similar to Reviewer 1 Major point 4. Please see our response there.

8. The authors have previously shown that the CST complex interacts with and recruits RAD51 to

stalled forks. Thus, despite their biochemical data, one could hypothesise that CST promotes fork stability indirectly via RAD51 stabilization on reversed forks and not by directly protecting them from MRE11-dependent nucleolytic digestion. The authors should directly assess RAD51 recruitment to stalled forks by SIRF and explicitly discuss this hypothesis based on these new results. This point may also help clarifying the epistatic relationship with BRCA2 in fork protection.

We performed RAD51 SIRF analysis and found that the binding of RAD51 at the stalled forks decreased (but not completely gone) in STN1 depleted cells (new Figure 7A). This result is consistent with our previous results showing that CST depletion attenuates RAD51 foci formation and RAD51 recruitment to GC-rich sites after fork stalling (Chastain et al, Cell Rep 2016; Wang, et al NAR 2018). We would like to clarify that in this manuscript, we do not exclude the possibility that CST may also recruit RAD51 to stalled forks to protect fork stability. The focus of this manuscript is to reveal a new mechanism that CST can bind to stalled forks and directly block MRE11 degradation of nascent strand DNA. The two protection mechanisms may co-exist. We have discussed the role of RAD51 in Discussion and modified our model (see the new Figure 7D).

9. As CST foci largely overlap with telomeres (Fig 4B) even upon HU treatment, it would be important to discriminate more specifically what structural determinants differently recruit CST or BRCA2 to stalled forks. Do BRCA2 and CST differentially localise at G4-forming (not only telomeric) regions experiencing replicative stress? While I realise that this aspect may be beyond the scope of this manuscript, the authors propose here an intriguing hypothesis. They should either remove it from the manuscript, or obtain at least some initial evidence to support it.

This is the hypothesis that we will be extremely interested in testing, but feel that it is beyond the scope of this manuscript. As suggested, we have removed the original Figure 6E.

MINOR POINTS:

1. Fig1A is unnecessary (at least as a main figure) and 1B could be easily incorporated in Fig 2.

We have replaced Figure 1 with new data in response to other reviewer's comments (Reviewer 1 major point 1). We also include all SIRF data from biological replicates in Supplemental Figure S1, which is a full page. We feel that it is better Figure 1 stands alone from Figure 2. We also think the schematic diagram in Figure 1A could help the readers to understand our experimental setting and results.

During revision, we have decided to combine part of the original Figure 2 and Figure 3 into one figure (revised Figure 2).

2. The panels 4A-C are controls for the key experiment in this figure (4D) and should be probably moved to the Supplementary figures. It is confusing for the flow of the manuscript to assess telomere recruitment in the main figure. In fact, it would be much more relevant to test directly recruitment to stalled forks of the delta700N mutant, which may nicely complement Fig. 4D in the main figure.

We use CST localization to telomeres as a supportive measure to show that Δ700N loses DNA binding ability. Figure 4D and 4E provides direct biochemical evidence that purified Δ700N loses DNA binding ability. In addition, unlike WT CTC1, Δ700N staining is diffused and fails to form foci after HU treatment. Together these evidence suggests that Δ700N does not bind to DNA and does not localize at stalled forks. We think that performing CTC1Δ700N SIRF analysis would be redundant.

3. Please add nucleotide markers in Fig 5 (DNA gels).

We now show the nucleotide markers in MRE11 degradation assay (new Supplementary Fig S4F).

4. BRCA2 downregulation efficiency should be shown by Western Blot.

BRCA2 western blot is now included in Supplemental Figure S6A.

5. A more absolute quantification of yH2AX staining should be used for Fig. 6C, using number of foci or mean intensity, assessing statistically significant differences between single and double knockout.

We have revised the graph and included it in the revised Figure 6C. We define nuclei containing >5 γH2AX foci as positive cells, and then compare the positive cells proportion among the different cells.

6. A quantitative measurement of colocalization should be done for the data in Fig. 6E.

Per the comment from this reviewer's Major point 9, we have removed this result.

7. A colour-code legend should be included in Figure S5 to discriminate amplifications, deletions, mutation etc.

We have updated the most recent available data in cBioPortal, and added the color-code legend. The new images are Included in the Supplemental Figure S8B.

8. Additional experimental details should be generally added to figure legends and/or Methods (e.g. statistical analysis in Fig. 5, timing of HU treatment in Fig 6E, antibodies used for SIRF, antibody dilutions used for the different experiments, ...).

We have included the detailed information as requested. Experimental details on statistical analysis and HU treatment conditions are included in Figure legends. Antibody information for SIRF, WB, IF, etc, including catalog numbers and dilutions, is included in Methods.

Referee #3:

This study by Lyu et al proposes a new function for the CST complex in preventing excessive nascent strand degradation at stalled replication forks, indicating an important role in maintain

genomic instability. Using a variety of experimental approaches, this study showed that CST localizes to stalled replication forks, therefore preventing persistent ssDNA accumulation and nascent strand degradation caused by MRE11 nuclease. Using DNA fiber analysis and biochemical systems, the author further imply CST binding and protection of reversed structures. Importantly, there is a spatial separation of CST with BRCA2, a major fork protection protein, implying alternative pathways.

Overall, the study is well designed and will undoubtedly add understanding to the replication genome stability field. The authors should however consider the following points for their revision.

1• The authors propose that the data in Fig 2C indicates a delay in replication recovery when CST is depleted. These data is weak, and RPA32 reduction could have several reasons. A more informative assay for replication recovery would be DNA fiber analysis.

> This is the same point as Reviewer 1 Minor point 7 and Reviewer 2 major point 2. Please see our response there.

2• It is not clear how CST protects from degradation. The authors mention a direct interaction with RAD51, the known actor for fork protection downstream of BRCA2. Is CST required for RAD51 foci formation or retention (stabilization)?

> The focus of this manuscript is to reveal a new mechanism that CST can bind to stalled forks and directly block MRE11 degradation of nascent strand DNA. We have previously shown that CST depletion results in a partial reduction of RAD51 foci formation after HU treatment (Chastain et al. Cell Rep 2016; Wang et al. NAR 2018). In the revised manuscript, we have provided new SIRF data showing that STN1 depletion diminishes (but not completely abolishes) RAD51 localization at stalled forks (Figure 7). We would like to clarify that in this manuscript, we do not exclude the possibility that CST may also recruit RAD51 to stalled forks to protect fork stability. The two protection mechanisms (RAD51-dependent and RAD51-independent) may co-exist. We have discussed the role of RAD51 in Discussion and modified our model (see the new Figure 7D). Please also see our response to Reviewer 1 Major point 2 and Reviewer 2 Major point 8.

3• The Brca2 antibody use for IF studies needs to be validated for specificity.

> We have provided BRCA2 western blot in Figure S7A. This is the same antibody used for our IF and also widely used in other publications (Alexandre, et.al., Nature, 2015; Kuntian, et.al., Genes & Development, 2016; Weiran, et.al., Nature Communication, 2017.). In addition, we provide IF images using BRCA2-null cell line PEO1 and its isogenic BRCA2+ cell line PEO4 to further validate the antibody specificity (new Supplemental Figure S6A).

4• The spatial separation of BRCA2 and CST is intriguing: is there a temporal or solely a spatial separation? E.g. are there more BRCA2 foci in early replicating cells compared to more CST foci in late replicating cells (consistent with CST's function at more difficult to replicate regions such

as telomers, which typically replicate late in S-phase). It also would be informative to show alternative methods.

We feel that elucidating the spatial relationship of BRCA2 and CST is beyond the scope of this manuscript. See our response to Reviewer 2 Major point 9.

5• SIRF assays need to be normalized to total Edu content, either by co-click with Alexa 488 or at a minimum by normalization slides with EdU-EdU PLA. It is likely that EdU concentrations increase with continued EdU exposure, and decrease with HU if it is degraded, and this will skew the results.

We have performed the EdU-EdU PLA experiments as suggested and provided the new results in Figure S1A. SIRF results that are included in the revised manuscript have been normalized by EdU-EdU PLA signals.

6• The biochemical assays require a negative control to show that the nuclease inhibition by CST is specific and not simple substrate titration availability. E.g. does SSB or RPA also block degradation in the system?

We now included RPA and bacterial ExoIII as control experiments to show that the nuclease inhibition by CST is specific (new Supplementary Fig S4A-D). Please see the detailed response to Referee 2, Major point 6.

7• Quantification of Fig 4B and 6E would be helpful.

Quantification has been included in the revised Figure 5A.

In response to the comment from Reviewer 2 Major point 9, we have removed Figure 6E from the revised manuscript.

- minor points

• The extent of fork protection seems only very mild- controls with BRCA2 knock down for direct comparison would be informative

Please see our response to Reviewer 1 Major point 4, which includes our fiber analysis of BRCA2 knockdown.

• LUC abbreviation is not explained in text

Explanation is added in Figure 2A legend.

• Fig3 E and 3F: include statistics to compare group1 (con) and group 3 con+SMARCAL/ZRANB3

Included. They are now in Fig 2G, 2F.

- at author's discretion:

CST depletion only mildly sensitizes cells to fork stalling agents and causes only a minor increase in micronuclei formation. Metaphase chromosome aberration assay may be more informative

We have performed metaphase chromosome aberration assay in shSTN1 and also in STN1/BRCA2 double-knockdown cells. New results are shown in Figure 6E.

1st Revision - Editorial Decision 24th Sep 2020

Thank you for submitting your revised manuscript to The EMBO Journal, and apologies for the delay in getting back to you with a decision. Two of the original reviewers have now assessed it once more, and generally found the work considerably improved towards becoming acceptable for publication. However, referee 1 still retains a number of reservations that may need experimental and/or presentational addressing. After further discussing these points with referee 2, I would not insist on further experiments in response to referee 1's point 1, while any additional controls in response to referee 1's point 2 would clearly be helpful for strengthening the study. With regard to referee 1's point 3 on the CST/BRCA2 relation, referee 2 also shares these concerns and the missing validation through DNA fiber assays. Although we realize that this aspect may not be a central part of the paper and its conclusions, we feel that in the absence of additional supporting data, any conclusion (and speculation) on the genetic relationship between CST and BRCA2 would have to be considerably toned down, acknowledging that this requires further molecular investigation.

I am therefore returning the manuscript to you for an exceptional second round of revision, to allow you to respond to and address the remaining major and minor points listed by the referees. During this additional round of revision, please also address the following important editorial points:

Referee #1:

While the authors have addressed many of my concerns and I applaud their efforts to revise the manuscript with the current difficulties created by the pandemic, there are still several concerns that have not been adequately addressed as well as additional points regarding the new data that should be addressed to fully support the major conclusions drawn from the study.

Major points:

1. The authors new data on the relationship between CST and RAD51 is intriguing. The authors interpret their current results to mean that RAD51-dependent fork reversal is required for CST to inhibit MRE11-dependent fork degradation. However, RAD51 is involved in both fork reversal and fork protection (Bhat et al. Cell Rep. 2018). Based on Fig. 7C, the levels of RAD51 siRNA depletion inhibit both activities. This experiment does rule out the possibility that it is the loading of RAD51 by CST and not MRE11 inhibition that leads to fork protection. This alternative model could also explain the results of CST depletion on fork protection throughout the paper (i.e. fork degradation, excess ssDNA, increased MRE11 at stalled forks). While the authors have pointed out this alternative means of fork protection by CST in the discussion, the conclusions of the manuscript are focused solely on a direct role of CST in MRE11 inhibition. It is possible that RAD51 loading by CST at reversed forks alone is responsible for fork protection. As such, I feel that the results, in their present form along with concerns raised in point #2, could be an overinterpretation of the in vitro studies. To address whether fork protection by CST is dependent on RAD51 loading at reversed forks, RAD51 levels could be modulated to inhibit RAD51-dependent fork protection but not fork reversal, described in Bhat et al. Cell Rep, 2018. If the author's model is correct, then fork

degradation with partial RAD51 depletion should be additive, if fork protection is dependent on MRE11 inhibition by CST, or only partially epistatic with CST depletion, if both models lead to fork protection, in CST depleted cells.

[Cross-referee comments from referee 2 / excerpt:
"...The experiment proposed may be tricky to do, as levels of RAD51 required for fork reversal and protection may have different modulations in different cell lines and may be difficult to control by partial siRNA-mediated downregulation. Possibly, a better experiment would be to test CST inactivation in cells containing a separation-of-function mutant of RAD51 (T131P), which has been shown to be permissive for fork reversal, but defective in fork protection (Mijic et al., Nat Comms 2017). However, I feel that this additional experimentation is not strictly required to support the claims included in the revised manuscript."]

2. While the new SIRF data with endogenous CTC1 and STN1 antibodies and the addition of replicate experiments address my concern about the localization of CST to replication forks, the specificity of the CTC1 antibody is a concern based on the blots shown in Figure S5. I am not entirely convinced that the band shown is CTC1 as the background bands are at different levels between lanes, suggesting loading may not be equal. (No loading control is shown.) This also put into question the levels of CTC1 knockdown by siRNA. Regardless, the numerous bands observed in the Western blot indicate that the antibody may not be suitable for PLA and suggest the possibility that another protein is being detected in the SIRF assay. The antibody to STN1 also appears to have several background bands (Fig. 2D), although much less than CTC1. A major point of the paper is that CST directly acts at stalled forks (as stated in the title, abstract and throughout the paper) so this point it critical to the authors' conclusions. Knockdown of CTC1 and STN1 or similar controls to demonstrate specificity of antibodies in the SIRF assay would greatly improve confidence in this result.

[Cross-referee comments from referee 2 / excerpt:
"Any additional controls on this set of experiments would be welcome as further support ... I see the possible concern of the reviewer on the reliability of the CTC1 antibody ... I don't think similar concerns can apply for STN1, also because the PLA seems - to my understanding - seems based on detection of the endogenous protein, and not of its flagged version (as in Fig. 2D)."]

3. There are three additional points regarding the CST/BRCA2 co-depletion that should be addressed: (1) The authors state in the abstract that CST inactivation induces synthetic lethality. Growth analysis has not been performed so it is unclear how their data supports a conclusion of synthetic lethality, which is that perturbation of two genes results in cell death whereas perturbation of a single gene results has little to no effect (Nijman FEBS Letters. 2011). Also, based on the EdU uptake in Fig. 6D, the effects are appear to be additive. (2) The authors have not addressed my previous comment that their data (micronuclei, anaphase bridge, gH2AX) appears to be additive. As currently written, the impression is given that loss of CST and BRCA2 causes a synergistic effect. However, their data would suggest that genetically CST and BRCA2 act in distinct pathways to promote genome stability. (3) The authors state that they were unable to successfully perform DNA fiber assays but were able to perform other genome instability assays (micronuclei, anaphase bridge, gH2AX). They provide data that this is due to detachment of cells with STN1/BRCA2 co-depletion. However, it is unclear, to this reviewer, how they avoided washing

the cells prior to fixation on coverslips for the genome instability assays and what is meant by all cells were collected for analysis. Is it possible that cells were also lost in these assays? Are the detached cells apoptotic? Clarification or additional data on these points are needed.

[Cross-referee comments from referee 2 / excerpt:
"As discussed in my own comments, I find it difficult to accept that the authors could not find experimental conditions to perform the fiber assays, early after co-depletion of the proteins. I also find it difficult to follow - form the technical point of view - how certain assays (micronuclei, anaphase bridges, etc.) are possible and used to support claims, while others (fiber assays) are deemed as not feasible. The authors have already acknowledged that fiber experiments would have been important to support key claims. I would encourage the authors to tone down any conclusion (and speculation) on the genetic relationship between CST and BRCA2, acknowledging that this requires further molecular investigation. That said, I don't think this is a central conclusion of the paper..."]

Minor Points:

1. It is not clear why the EdU-SIRF signal is higher in the HU controls in Fig. S1E (and Fig S1A?). The methods do not state when HU was added but I presume that it was after the 8 min EdU label and that EdU was washed out prior to HU addition.

2. Why does the relative BrdU intensity in the new Fig 2B differ so much from the previous Fig 2B?

3. The y-axis in Fig 2H needs correction.

Referee #2:

The authors have done overall a good job addressing key concerns from this and other reviewers, and the manuscript now has much stronger support to its key claims. I remain of the opinion that, regardless of the results, proper fork restart DNA fiber experiments would have been an important addition to the story.

The authors have provided new technical arguments on why possible synthetic effects of CST and BRCA2 in DNA fibers could not be effectively measured. I am not entirely convinced that all possible attempts to perform these important experiments have been done (e.g. analysing cells at earlier time points after siRNA transfection). However, I can accept that they are currently unable to test this directly. Also, this point is now extensively and fairly discussed in the Results, and there are no dogmatic statements on this open point in Discussion.

I feel this is a much improved manuscript, which makes a relevant contribution to the field of DNA replication stress, in the increasingly popular area of replication fork protection.

Point-by-point Response:

Referee #1:

While the authors have addressed many of my concerns and I applaud their efforts to revise the manuscript with the current difficulties created by the pandemic, there are still several concerns that have not been adequately addressed as well as additional points regarding the new data that should be addressed to fully support the major conclusions drawn from the study.

Major points:

1. The authors new data on the relationship between CST and RAD51 is intriguing. The authors interpret their current results to mean that RAD51-dependent fork reversal is required for CST to inhibit MRE11-dependent fork degradation. However, RAD51 is involved in both fork reversal and fork protection (Bhat et al. Cell Rep. 2018). Based on Fig. 7C, the levels of RAD51 siRNA depletion inhibit both activities. This experiment does rule out the possibility that it is the loading of RAD51 by CST and not MRE11 inhibition that leads to fork protection. This alternative model could also explain the results of CST depletion on fork protection throughout the paper (i.e. fork degradation, excess ssDNA, increased MRE11 at stalled forks). While the authors have pointed out this alternative means of fork protection by CST in the discussion, the conclusions of the manuscript are focused solely on a direct role of CST in MRE11 inhibition. It is possible that RAD51 loading by CST at reversed forks alone is responsible for fork protection. As such, I feel that the results, in their present form along with concerns raised in point #2, could be an overinterpretation of the in vitro studies. To address whether fork protection by CST is dependent on RAD51 loading at reversed forks, RAD51 levels could be modulated to inhibit RAD51-dependent fork protection but not fork reversal, described in Bhat et al. Cell Rep, 2018. If the author's model is correct, then fork degradation with partial RAD51 depletion should be additive, if fork protection is dependent on MRE11 inhibition by CST, or only partially epistatic with CST depletion, if both models lead to fork protection, in CST depleted cells.

[Cross-referee comments from referee 2 / excerpt:
"...The experiment proposed may be tricky to do, as levels of RAD51 required for fork reversal and protection may have different modulations in different cell lines and may be difficult to control by partial siRNA-mediated downregulation. Possibly, a better experiment would be to test CST inactivation in cells containing a separation-of-function mutant of RAD51 (T131P), which has been shown to be permissive for fork reversal, but defective in fork protection (Mijic et al., Nat Comms 2017). However, I feel that this additional experimentation is not strictly required to support the claims included in the revised manuscript."]

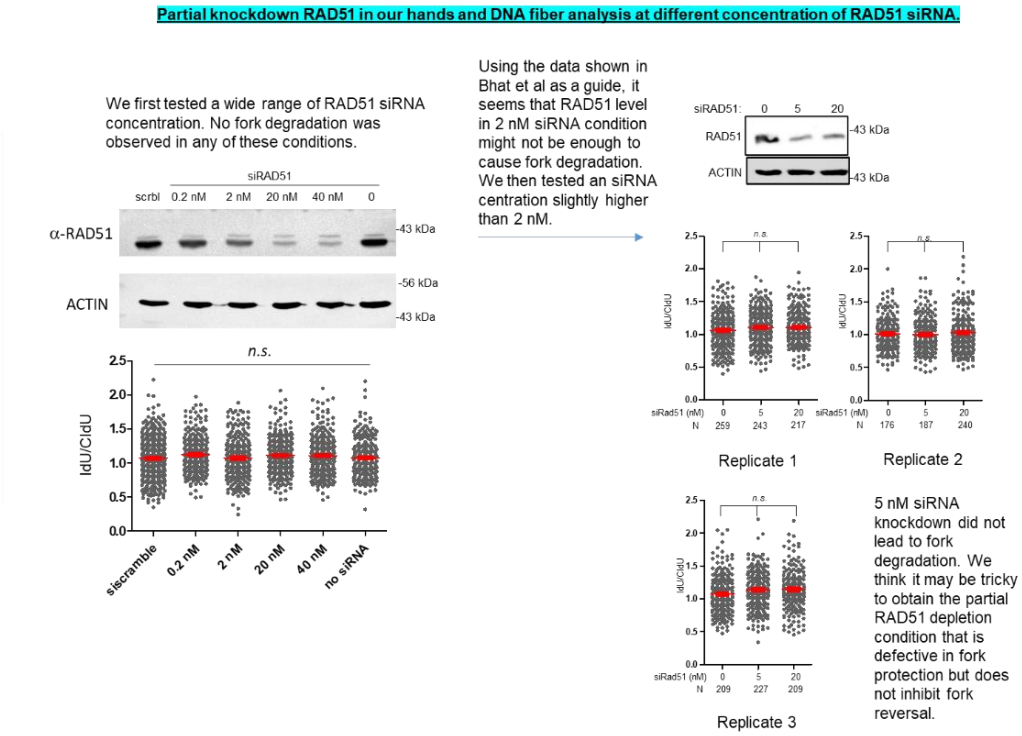
The point from Reviewer 1 is that fork degradation observed in CST deficient cells can be explained solely by the RAD51-dependent mechanism rather than loss of MRE11 blocking. Again, we thank the reviewer for the suggestion of doing the CST and RAD51 double knockdown. We have given it careful consideration. We think it would be very challenging to clearly address this question using cell-based approaches. Here's why:

There are three important facts: (1) RAD51 binds to forks before fork reversal and after fork reversal. (2) We know CST depletion attenuates RAD51 recruitment to stalled forks, but we do not know whether it affects RAD51 recruitment pre-fork reversal or after reversal, or both. (3) CST depletion reduces RAD51 recruitment but does not completely abolish its recruitment. In a way, CST depletion is like a partial RAD51 knockdown. With these three facts in mind, we would like to discuss the possible outcomes from the proposed CST/partial RAD51 double knockdown experiment. If CST affects pre-reversal RAD51 recruitment, depleting CST in RAD51 partial knockdown cells may

further reduce RAD51 at pre-reversal forks to the level that fork reversal is inhibited. Then no fork degradation would be seen. Therefore, the result would not provide any insight on whether CST directly blocks MRE11. If CST affects RAD51 recruitment after fork reversal, then the nascent strand degradation observed in CST depleted cells should include the combined effects from reduced RAD51 at reversed forks AND loss of MRE11 block. Since RAD51-dependent fork protection is already defective in CST knockdown cells, further reducing RAD51 most likely would not yield additive effects. This would be a negative result that is difficult to interpret.

In our opinion, while biochemical analysis is not perfect, it uses purified proteins and provides a defined system showing that the CST complex is able to block MRE11 degradation. Ultimately, the best way to address this question is perhaps to use a separation-of-function CST mutation that abolish CST/RAD51 interaction but still permits CST binding to DNA, so that RAD51 recruitment is defective but CST still binds DNA. However, obtaining such a separation-of-function mutation is not a trivial task.

We did try different concentrations of RAD51 siRNA in an attempt to control the RAD51 partial knockdown in order to inhibit RAD51-dependent fork protection but not fork reversal. The data published in the paper referred by the reviewer is copy/pasted in the figure below (left panel). Our data are shown in the middle and right panels. While we could partially deplete RAD51 at 2 nM siRNA (middle panel), we did not observe fork degradation using a wide range of siRNA concentrations from 0.2 to 40 nM (middle panel). Since our 2 nM siRNA lane still had considerable amount of RAD51, we thought perhaps this concentration was not high enough. Using the western blot image published in Bhat et al as a guide (left panel), we then slightly increased siRNA to 5 nM, hoping to reproduce the published data. Unfortunately, we did not observe fork degradation (right panel). We agree with Referee 2 that it is very tricky to control the RAD51 level that can lead to defective fork protection but does not inhibit fork reversal.



2. While the new SIRF data with endogenous CTC1 and STN1 antibodies and the addition of replicate

experiments address my concern about the localization of CST to replication forks, the specificity of the CTC1 antibody is a concern based on the blots shown in Figure S5. I am not entirely convinced that the band shown is CTC1 as the background bands are at different levels between lanes, suggesting loading may not be equal. (No loading control is shown.) This also put into question the levels of CTC1 knockdown by siRNA. Regardless, the numerous bands observed in the Western blot indicate that the antibody may not be suitable for PLA and suggest the possibility that another protein is being detected in the SIRF assay. The antibody to STN1 also appears to have several background bands (Fig. 2D), although much less than CTC1. A major point of the paper is that CST directly acts at stalled forks (as stated in the title, abstract and throughout the paper) so this point it critical to the authors' conclusions. Knockdown of CTC1 and STN1 or similar controls to demonstrate specificity of antibodies in the SIRF assay would greatly improve confidence in this result.

[Cross-referee comments from referee 2 / excerpt:
"Any additional controls on this set of experiments would be welcome as further support ... I see the possible concern of the reviewer on the reliability of the CTC1 antibody ... I don't think similar concerns can apply for STN1, also because the PLA seems - to my understanding - seems based on detection of the endogenous protein, and not of its flagged version (as in Fig. 2D)."]

We have performed CTC1 SIRF using knockdown cells and included the results in Appendix Fig S1F. Knocking down CTC1 dramatically reduced CTC1 SIRF foci, suggesting that the observed CTC1 SIRF signal is specific. We have noticed that the CTC1 antibody quality varies greatly from batch to batch. To minimize such variation, the new SIRF experiments were performed using the same antibody batch used in the experiment shown in the main Fig 1. In addition, this new SIRF experiment was conducted by a new lab member. He also analyzed the data. Given that our SIRF data can be reproduced independently by two individuals, we are highly confident that CST localizes at stalled forks.

In case the reviewer wonders why the CTC1 antibody detects so many “non-specific” bands on western blot but SIRF looks much cleaner, we think this may be due to the differences in how western and SIRF are performed. We used whole cell lysates in western blot, whereas in SIRF experiments we pretreated cells with Triton prior to cell fixation and antibody incubation. This pretreatment step, adopted from the published telomere protein staining protocol (Miyake et al Mol Cell 2009, https://doi.org/10.1016/j.molcel.2009.08.009; Huang et al. Exp Cell Res 2017 https://doi.org/10.1016/j.yexcr.2017.03.058), removes some if not the majority of non-chromatin-bound proteins prior to cell fixation. (Note that even with pretreatment, we still noticed substantial SIRF signal outside nuclei). Only nuclear SIRF foci were included in quantification. In contrast, western blots detect all nuclear and non-nuclear proteins. Thus, it is possible at least some non-specific western bands detected by the CTC1 antibody are cytosolic proteins present in the whole cell lysates.

Thanks for pointing out the “lack of” loading control in Fig S5A (ii). The loading control for Appendix Fig S5A (ii) is the same as the actin control in Fig S5A (i). Basically, Westerns in Fig S5A (i) and S5A (ii) were performed in parallel using the same cell lysates. Equal amounts of lysates were loaded on a regular 8% SDS-PAGE gel and a 4-15% gradient gel. Gels were run side-by-side and western was performed at the same time. Thus, only one loading control was done. We have copy/pasted the loading control from (i) to (ii), and also clarified it in figure legends.

3. There are three additional points regarding the CST/BRCA2 co-depletion that should be addressed: (1) The authors state in the abstract that CST inactivation induces synthetic lethality. Growth analysis has not been performed so it is unclear how their data supports a conclusion of synthetic lethality, which is that perturbation of two genes results in cell death whereas perturbation of a single gene results has little to no effect (Nijman FEBS Letters. 2011). Also, based on the EdU uptake in Fig. 6D, the effects are appear to be additive. (2) The authors have not addressed my previous comment that their data (micronuclei, anaphase bridge, gH2AX) appears to be additive. As currently written, the

impression is given that loss of CST and BRCA2 causes a synergistic effect. However, their data would suggest that genetically CST and BRCA2 act in distinct pathways to promote genome stability. (3) The authors state that they were unable to successfully perform DNA fiber assays but were able to perform other genome instability assays (micronuclei, anaphase bridge, gH2AX). They provide data that this is due to detachment of cells with STN1/BRCA2 co-depletion. However, it is unclear, to this reviewer, how they avoided washing the cells prior to fixation on coverslips for the genome instability assays and what is meant by all cells were collected for analysis. Is it possible that cells were also lost in these assays? Are the detached cells apoptotic? Clarification or additional data on these points are needed.

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For micronuclei, anaphase bridges, γH2AX, BrdU incorporation assays, we seeded cells on cover slips (placed inside a 24-well dish) and performed siRNA transfection on cover slips. After RNAi, media were very carefully removed to avoid losing too many cells, and then paraformaldehyde was added to directly fix cells on cover slips without PBS pre-washing. Of course, floating (dead) cells were removed, but there were enough cells left on cover slips to continue the analysis. Since cells were fixed on cover slips, they stayed through the following procedures. In contrast, the DNA fiber assay requires extensive washing to remove CldU and IdU prior to cell collection. Any cell that cannot survive extensive washing is removed.

We have toned down the speculation of the genetic relationship between CST and BRCA2 in this revision. Specifically, we changed “synthetic lethal” to “additive” throughout the manuscript in Abstract, and Results. We also removed our speculation in “Discussion” on Page 17 starting from “Given that CST prefers binding to DNA……”, and replaced it with one sentence: “Further molecular investigation is needed to pinpoint the genetic relationship between these two important genome maintenance players.”

Minor Points:

1. It is not clear why the EdU-SIRF signal is higher in the HU controls in Fig. S1E (and Fig S1A?). The methods do not state when HU was added but I presume that it was after the 8 min EdU label and that EdU was washed out prior to HU addition.

We removed EdU by washing prior to HU treatment. We have now included this description in “Materials and Methods”.

The higher EdU-SIRF signals in HU treated controls observed in our experiments are consistent with other’s observations (Roy et al. J Cell Biol, 2018, Supplemental Figure S1). While it is unclear why EdU-SIRF signal is higher in the +HU control, we think this could be explained by the way EdU labeling is conducted. In the SIRF experiment, high concentration of EdU (125 μM) is used in the 8 min pulse labeling. In untreated samples, cells are fixed immediately after EdU pulse labeling. Thus EdU incorporation stops at 8 minutes. In contrast, HU-treated cells are allowed to grow for additional 3 hours after EdU removal. Despite EdU is washed out from media, the residual EdU inside the cells can still be incorporated into genome in the first few minutes of HU treatment since it takes time for nucleotide pool

to be depleted and fork stalls. This additional EdU incorporation can lead to increased EdU-SIRF signals.

2. Why does the relative BrdU intensity in the new Fig 2B differ so much from the previous Fig 2B?

Our lab moved from the west coast to the current institution in Chicago while the manuscript was under review. Results in previous Fig 2B were obtained at the former institution. Data in the new Fig 2B were acquired at our current institution. After moving, we have obtained a new Zeiss fluorescent microscope with new filtersets, new objectives, new camera, etc. The new microscope is perhaps more sensitive in detecting fluorescence signals. Reagents and supplies were also changed. It is highly likely that the discrepancy is due to the changes in equipment and experimental settings at the two different locations.

3. The y-axis in Fig 2H needs correction.

We could not find anything wrong with the y-axis in Fig 2H. We also checked other figures and did not find mistakes in y axis of any figure.

Referee #2:

The authors have done overall a good job addressing key concerns from this and other reviewers, and the manuscript now has much stronger support to its key claims. I remain of the opinion that, regardless of the results, proper fork restart DNA fiber experiments would have been an important addition to the story.

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Thank you!

2nd Revision - Editorial Decision 20th Oct 2020

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 ↓

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Corresponding Author Name: Weihang Chai
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Manuscript Number: EMBOJ-2019-103654

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?	Sample size for each experiment is shown in the figures or figure legends. Sample size was based on standard practice in the field and was large enough to peform statistical analysis.
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?	All the collected data have been analysed.
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.	For each experiment we performed two or three biological replicates, and for key experiments two individual research scientists performed experiments independently to validate the results.
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.	NA
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. Statistical tests are mentioned in the figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical analyses were performed with one-way ANOVA analysis with post hoc Tukey when more than two groups were assessed, and Mann Whitney test when two groups were assessed. Specific tests used for each experiment are described in the figure legends.
Is there an estimate of variation within each group of data?	NA

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Is the variance similar between the groups that are being statistically compared?	NA

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).	Described in the Materials and Methods section
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Described in the Material and Methods section, and all the cells have tested to exclude mycoplasma 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
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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).	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

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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