LRR1-mediated replisome disassembly promotes DNA replication by recycling replisome components

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Review Timeline:

- Submission Date: 2020-09-22
- Editorial Decision: 2020-10-23
- Revision Received: 2021-03-30
- Editorial Decision: 2021-04-27
- Revision Received: 2021-04-30

Monitoring Editor: David Gilbert

Scientific Editor: Melina Casadio

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202009147
1st Editorial Decision

October 23, 2020

Re: JCB manuscript #202009147

Prof. Tobias Meyer
Stanford University
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Dear Prof. Meyer,

Thank you for submitting your manuscript entitled "LRR1-mediated replisome disassembly promotes DNA replication by recycling replisome components" and thank you for your patience with the peer review process. Your manuscript has been assessed by expert reviewers, whose comments are appended below. Although the reviewers express potential interest in this work, significant concerns unfortunately preclude publication of the current version of the manuscript in JCB.

You will see that the reviewers felt that the study is of interest and very carefully executed, and two agree that it addresses an important gap in the field and presents data of high interest to replication and cell cycle experts. However, they also felt that the model is not supported without data exploring whether the slower replication phenotype is due to a failure to recycle replisome components upon LRR1 loss. Revs#2 and #3 point out that other mechanisms could be at work. We find the reviewers' expert comments constructive and agree that the conclusion that the slow replication is due to failure to recycle replisome components is premature and should be toned down. As highlighted by the referees, cell fractionation for CDC45 abundance needs to be done. We encourage you to focus efforts in revision to address this gap and experimentally tackle the reviewers' suggestions, including Reviewer #2's major points. Reviewer #2's 'other comments' could be addressed textually. We would not suggest prioritizing experiments in other cell lines but rather deeper experiments in MCF10A cells.

Please let us know if you are able to address the major issues outlined above and wish to submit a revised manuscript to JCB. Note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit the spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
During replication termination, CMG and associated leading strand replication factors are removed from DNA in a regulated process that involves poly-ubiquitylation of CMG on its Mcm7 subunit and subsequent CMG disassembly by p97 ATPase. Ubiquitylation of Mcm7 in interphase is dependent on SCFDia2 in yeast and CRL2Lrr1 E3 ubiquitin ligase in worms and Xenopus egg extracts. The identity of the E3 ubiquitin ligase that regulates this process as well as potential consequences of mis-regulation of CMG unloading in human cells are unknown.

The manuscript entitled "LRR1-mediated replisome disassembly promotes DNA replication by recycling replisome components" reports that LRR1 is an essential gene and that human cells
lacking LRR1 accumulate replisome components on DNA in S phase, which is accompanied by a suppression of DNA replication. Fan and colleagues propose that the latter phenomenon can be explained by sequestration of rate-limiting replication factors on DNA due to impaired disassembly and recycling of replisome components. Furthermore, Fan et al. show that LRR1 deficiency leads to an arrest in G2 phase of the cell cycle, which can be overcome by inhibition of the ATR signaling.

The observation that loss of Lrr1 correlates with a progressive accumulation of replisome components on chromatin, slowing of DNA replication, and failure to complete mitosis is fascinating and will interest many in the replication and cell cycle fields. For the most part, the experiments are executed to a very high standard, and the imaging in particular is impressive. The only limitation is that the experiments presented cannot prove that the reason for slower replication is failure to recycle replisome components. This would have required rescuing the rate of replication via overexpression of replisome factors. Notably, overexpression of CDC45 or GINS did not rescue replication in the absence of Lrr1, but of course, many factors could be limiting, and so this experiment will require a long term investment of time. In the meantime, to strengthen their conclusion, it would be useful to show that Lrr1 depletion leads to depletion of CDC45 and GINS in the nucleoplasm (see comment 1). Even with this experiment, the conclusion is still tentative. Therefore, throughout the paper, the authors should soften their language that failure to terminate slows replication, and in the Discussion, they should present alternative interpretations of their data.

**COMMENTS:**

1. Fan et al show that the loss of LRR1 induces accumulation of CDC45 on chromatin during S phase. They also show that this increase was not accompanied by "a corresponding increase in the total pool of CDC45, but rather by a slight decrease". The accumulation of CDC45 on chromatin is also negatively correlated with the efficiency of DNA replication in LRR1 knockdown cells. Based on this, the authors conclude that the observed defect in DNA replication is caused by depletion of rate-limiting soluble DNA replication factors. However, the authors do not show that these replication factors, e.g. CDC45, are depleted from the nucleoplasm. To further support their interpretation, the authors should perform a cell fractionation and western blot analysis of CDC45 levels in the cytoplasm, nucleoplasm, and chromatin fractions in G1 phase and in S phase. If all CDC45 is indeed trapped on DNA, there should be a noticeable decrease in CDC45 levels in soluble nucleoplasm in LRR1-deficient cells in S phase in comparison to G1 phase and control cells.

2. Why does LRR1 knockout have a very subtle effect on the EdU incorporation in Figures 2H and S2H in comparison to other experiments?

3. The authors show that LRR1 loss leads to activation of ATR signaling in G2/M phase. The authors state: "failure to unload CMG helicases after LRR1 loss triggers a G2/M checkpoint that activates the ATR/Chk1/Wee1 pathway, arresting cells in G2 and blocking mitosis". However, the G2 arrest could be a result of failure to modify other Lrr1 targets. This should be discussed.

4. In Figure 2A-B, were cells fixed 30 hours after release from serum starvation or 6 hours after release? The legend is not clear on this. 30 hours would be a long time. Irrespective of the time, how do the authors know the cells entered S phase with similar kinetics in the two genotypes?

**MINOR COMMENTS:**
1. Authors use "normalized to sgCNTL" values for showing quantification of the incorporated EdU signal for Figure 2B, 2H, S2B, S2H, but "R.F.U" values for Figure 2E, S2E, S2G, and S2I. It would be better to use the same units and scales for all these panels for the easiest comparison of these experiments.

2. Figure S4G panel is referred in the text prior to panels S4E and S4F, and therefore, the order of panels should be changed accordingly.

3. Can the authors speculate about the trigger of ATR pathway activation in the absence of LRR1? Do they think that some replication forks eventually stall when neighboring origins fail to fire?

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, authors examined the role of the E3 ubiquitin ligase CRL2LRR1 in human cells. Authors showed that LRR1-depleted cells accumulate Cdc45 and other replisome components on chromatin and exhibit reduced level of DNA synthesis. They concluded that the accumulation of replisome factors on chromatin due to failure of replisome disassembly inhibits recycling of limited replication factors, thus reducing the level of DNA synthesis. They further show that the slowed/compromised replication (or accumulated replication factors?) activates ATR-Chk1-Wee1 checkpoint, inhibiting M phase progression. Finally they showed that LRR1 is required for proliferation of the mammalian cells.

Generally, the experiments are conducted very carefully. The data presented are convincing and are consistent with the authors' conclusions.

Authors propose that continuous disassembly of replisome, as it terminates during the course of S phase progression, is required for efficient S phase progression. This is an interesting possibility but has not been rigorously examined in the manuscript.

I would like to suggest the authors address following points to clarify some unsolved issues and to further improve the manuscript.

Major comments

1. Proteins abundance is examined only by the staining or FACS. The protein level, especially that of Cdc45, needs to be examined by western blotting after cell fractionation. According to the authors' conclusion, the Cdc45 protein level in the detergent soluble fractions after knockout of LRR1 should be reduced. However, this has not been directly shown. The increased staining signals on chromatin most likely reflects the increased level of the protein, but it could be that Cdc45 may be refractory to the detergent extraction, or the antibody epitope may be more accessible due to the change of replisome structure after LRR1 depletion.

2. Would it also be possible that the only the chromatin-bound Cdc45 is stabilized by LRR1 depletion? This could be examined also by western blotting analyses of the stability of Cdc45 in detergent-soluble and -insoluble fractions.

3. Although the authors' proposition that the block of the recycling of replication factors inhibits DNA synthesis in LRR1 depleted cells is consistent with the results, it would be also possible that
the Cdc45 remaining on the chromatin may be actively inhibiting the process of DNA replication. DNA fiber analyses should be conducted to examine if the fork rate is reduced and/or new origin firing is reduced in LRR1 depleted cells. This will give information regarding how DNA synthesis is inhibited in the absence of LRR1. Related to this, I would also like to see cell cycle distribution of LRR1-depleted cells by simple FACS of PI-stained cells.

4 Authors show that LRR1 depletion activates ATR-Chk1-Wee1 checkpoint. Is Chk1 activated (S317 or S345 phosphorylation of Chk1) after LRR1 knockout? Are single-stranded DNA segments accumulated in the absence of LRR1? Does RPA phosphorylation increase? Are DNA damages induced (gamma-H2AX signals)? Examination of these issues would give some insight into what kind of replication stress is generated after LRR1 depletion. (Is it the arrested replication fork, or slowed replication fork, or the incomplete DNA replication [the presence of unreplicated segments]? ) DNA fiber analyses, suggested in my comment #3, may also give some clue to these issues.

Other comments

5. Authors state that "it is plausible that as a result of increased origin firing and higher demand for replication factors, cancer cells might be more sensitive to reduced levels of rate-limiting replisome components upon loss of CRL2LRR1 activity and sequestration of replisome components on chromatin." This is an interesting possibility but authors only examined one cancer cell line in this manuscript. I would like to know if LRR1 regulates replication termination in non-cancer cells in a manner similar to that operated in cancer cells, since, generally, non-cancer cells have much lower levels of replication factors (including Cdc45 and Cdc7, normally rate-limiting factor for replication initiation, and helicase component Mcm) compared to cancer cells, and may adopt a different strategy to deal with disassembly of replisome. I would appreciate if authors could add any information or discussion regarding this.

6. It appears that the EdU pattern in LRR1 depleted cells represent mostly that of mid-S/late-S replicating cells (e.g. Figure 2A). Are these statistically significant? There are also cells that are positive for Cdc45 but are not replicating. In what cell cycle stage are these cells present?

7 The Cdc45 staining signals are very strong in LRR1 depleted cells. They form rather strong nuclear foci. I wonder if these foci represent the replication termination/ or pause sites where Cdc45 needs to be disassembled during normal S phase. I felt it would be interesting to determine by ChIP-seq where Cdc45 is bound on chromatin under this condition. I would not request authors to conduct the experiments, but appreciate if they have any thought on this.

Reviewer #3 (Comments to the Authors (Required)):

In this study by Fan et al, the authors have characterized the replication and proliferation phenotypes of MCF10A cells lacking the LRR1 Cullin adapter. The principle findings are that LRR1-deficient cells retain the CMG helicase on DNA - particularly in late S phase. These cells are also severely compromised for DNA synthesis rate as measured by EdU incorporation during a pulse label. The experiments are highly quantitative image analyses of single cells. There are several strengths of the experimental approach including the analysis of cells acutely depleted of LRR1 within a single cell cycle and the excellent use of a complementing LRR1 construct. The experiments themselves are convincing, and the manuscript is clearly presented.
I have two primary concerns: the significance of the advance and the support for claims and conclusions in the manuscript. I also have some comments on experiments and presentation at the end.

1. Significance. This study is a reasonably thorough characterization of one human cell line, but the unloading defect is expected from several other studies in yeast, worms, and frog oocytes. In this regard, it is confirmation of conservation across species of the role of LRR1 in CMG unloading. The possible novelty here is the correlated defect in the rate of DNA synthesis in human cells which was not noted in the frog model, but is consistent with the S phase accumulation in dia2-null yeast (Blake 2006). Similarly, the transcriptome analysis is well done, but there are a great many datasets already for palbociclib-sensitive genes in many cell lines (perhaps not MCF10A). The focused sgRNA screen is a nice and relatively unbiased approach to finding new cell cycle genes, but not particularly new; it seems one could have started from existing datasets to get to a similar group of 30 genes in S1B.

2. Author Conclusions. There are claims that are not fully supported by the available data:
   a. In the abstract itself, "cells disassemble active replisomes to prevent re-replication and genome instability." There are no experiments here to address genome instability or re-replication, and the one published experiment in worms suggests but does not directly prove re-replication as the cause of increased DNA content. In fact, the first sentence in the abstract is the only time re-replication/rereplication appears in the document.
   b. Importantly, the authors frequently escalate a correlation they show into a causal relationship. In particular, the fact that LRR1 loss leads to some replication proteins retained on chromatin and to slower DNA synthesis does not automatically meant that they have established retained CMG as the sole or primary cause of the EdU phenotype. LRR1 is a substrate adaptor for an enzyme that almost surely has more substrates than the MCM complex. All of the other substrates would be perturbed in these knockout cell lines, and one or more of them could be the cause of or contribute to a general S phase slowdown. While the model the authors suggest is consistent with their data, they can't claim to have determined the mechanism of DNA synthesis inhibition. To make that claim, they would need to do more than establish the correlation. For example, preventing CMG removal by some other means in LRR1-positive cells should have the same phenotype as the LRR1-negative cells.
   c. The authors also claim to have established that the retention of CMG on chromatin sequesters initiation factors that would normally be recycled. Page 6: "Unexpectedly, we show that the failure to unload CMG helicases increasingly slows down DNA replication by sequestering rate-limiting replisome components on chromatin and blocking their recycling."

While it is true that limiting initiation factor recycling is thought to be an important contributor to the replication timing program, there are no experiments here that show the retained components are limiting. The one observation that supposedly supports this notion is Figure 2F. The authors seem to predict that retaining CMG should lead to "a corresponding increase in the total pool of CDC45..." (page 5, lines 11-13); this implies that they consider LRR1 to promote CMG or Cdc45 degradation counter to other studies of LRR1 orthologs and their own staining data? It is already well known that polyubiquitylation of MCM7 in model systems does not induce degradation, and the experiments here do not show changes in levels dependent on LRR1 in human cells. One might expect an increase in the soluble fraction relative to chromatin, but that was not measured. The fact that there was not much change in levels is not sufficient evidence for a defect in recycling or sequestering limiting components. They attempted to suppress the phenotype by single protein overproduction (Cdc45 or one subunit of GINS) to no effect. In truth, tests of limiting initiation components have typically needed to overproduce four proteins. Therefore, while the sequestering
idea is possible, the authors cannot claim to have demonstrated this as the mechanism.

Moreover, the authors have not considered alternative explanations beyond testing checkpoint dependence. Retained complexes might affect fork speed or create collisions with replication or transcription complexes, etc.

All-in-all, these shortfalls mean the current study doesn't actually establish the mechanism of the DNA synthesis defect which is the main novelty. That leaves the documentation of the phenotype the primary advance which seems too thin at this point.

Additional remarks:
i) There is no analysis of MCM7 polyubiquitination which is important to establish that LRR1 is the primary E3 adaptor in these cells.
ii) There is no analysis of LRR1 protein in this study. These assays would establish LRR1 protein as a growth/cycle-regulated protein as well as E2F-regulated gene and confirm the knockout success and no alternative Lrr1 protein production.
iii) All experiments are confined to a single cell line with the exception of data mining. It would be improved to test the principle findings in at least one other line.
iv) What is the fate of LRR1-negative cells that divide in the presence of wee1 inhibitor? Does that completely rescue proliferation or are cells arriving in G1 with under-replicated DNA? If the latter, then unreplicated DNA might be both the activator of the G2/M checkpoint and the source of long-term proliferative failure.
v) Many bar graphs are data from one single experiment treating each single cell as a replicate. Given JCB's endorsement of more rigorous comparison of biological replicates (Lord et al JCB 2020), the authors should consider ways to show reproducibility across separate experiments instead of variation within one experiment. The good examples are 2H, 3D etc. Adding lines for the 25th and 75th percentile that look exactly like statistical representations of variation such as SD or SEM is confusing and should be presented differently (e.g. box-and-whiskers).
vi) The Discussion is largely a recap of the results and doesn't add much context or new concepts. Consider a discussion of alternatives, mechanisms, study limitations, what the wee1 pathway is activated by, etc.
Reviewer #1 (Comments to the Authors (Required)):

During replication termination, CMG and associated leading strand replication factors are removed from DNA in a regulated process that involves poly-ubiquitylation of CMG on its Mcm7 subunit and subsequent CMG disassembly by p97 ATPase. Ubiquitylation of Mcm7 in interphase is dependent on SCFDia2 in yeast and CRL2Lrr1 E3 ubiquitin ligase in worms and Xenopus egg extracts. The identity of the E3 ubiquitin ligase that regulates this process as well as potential consequences of mis-regulation of CMG unloading in human cells are unknown.

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The observation that loss of Lrr1 correlates with a progressive accumulation of replisome components on chromatin, slowing of DNA replication, and failure to complete mitosis is fascinating and will interest many in the replication and cell cycle fields. For the most part, the experiments are executed to a very high standard, and the imaging in particular is impressive. The only limitation is that the experiments presented cannot prove that the reason for slower replication is failure to recycle replisome components. This would have required rescuing the rate of replication via overexpression of replisome factors. Notably, overexpression of CDC45 or GINS did not rescue replication in the absence of Lrr1, but of course, many factors could be limiting, and so this experiment will require a long term investment of time. In the meantime, to strengthen their conclusion, it would be useful to show that Lrr1 depletion leads to depletion of CDC45 and GINS in the nucleoplasm (see comment 1). Even with this experiment, the conclusion is still tentative. Therefore, throughout the paper, the authors should soften their language that failure to terminate slows replication, and in the Discussion, they should present alternative interpretations of their data.

Thank you for the comments. We have performed new experiments, including subcellular fractionation and rescue experiments, that provide support for our working model. We have also expanded the Discussion to include alternative interpretations of the data.

- Subcellular fractionation of replisome components
  To measure the abundance of replisome components in the soluble pool, we performed subcellular fractionation on control and LRR1 knockout cells. We synchronized serum-released cells with aphidicolin to block cells at the G1/S transition, after which we released cells from the
block for 5 hours and harvested the cells (Figure 3B). We fractionated cell lysates into detergent (0.5% Triton X-100)-soluble and insoluble pools and found that LRR1 knockout reduced the levels of CDC45, POLE1, and Timeless in the soluble pool (Figures 3C-D), and this reduction is dependent on progression through S phase (Figures S4B-D). We note that as LRR1 knockout was performed using transient sgRNA transfection, the knockout was not complete (successful in 52.0% of cells). As a result, the bulk-cell measurement likely underestimates the true extent of depletion in the soluble pool. We also found that CDC45, POLE2, and Timeless were all exclusively nuclear localized (Figure S4E), suggesting that the detergent-soluble fraction of these replisome components reflects the nucleoplasmic pool rather than cytoplasmic pool. These results suggest that LRR1 knockout indeed reduces the level of replication factors from the soluble pool, which supports our working model that the slower DNA replication is due to a failure to recycle replisome components into the soluble pool after LRR1 loss.

- Rescuing reduced rate of DNA replication with E2F hyperactivation

The depletion of multiple replisome components from the soluble pool after LRR1 knockout suggests that overexpression of single components is unlikely to rescue slowed DNA replication. We agree that trying to find the combination of factors that are rate-limiting is unpractical. Thus, we took an alternative approach. We argued that as the vast majority of replisome components (including CDC45, GINS1-4, POLE1, POLE2, Timeless, TIPIN, Claspin, WDHD1) are E2F transcriptional targets (Supplemental Table 3 and 4), upregulating E2F activity may increase the expression of many replisome components at the same time. We knocked down E2F6, E2F7, and E2F8, which suppress E2F-responsive genes (Chen et al., 2009), and confirmed elevated levels of CDC45, Timeless, and POLE2 in S phase (Figure S4G). In cells transfected with control sgRNA, knocking down E2F6/7/8 did not significantly increase the rate of DNA replication; in cells where LRR1 were successfully knocked out, E2F6/7/8 knock down partially rescued slowed DNA replication (Figure 3G). This rescue experiment provides another piece of evidence supporting our working model.

COMMENTS:

1. Fan et al show that the loss of LRR1 induces accumulation of CDC45 on chromatin during S phase. They also show that this increase was not accompanied by "a corresponding increase in the total pool of CDC45, but rather by a slight decrease". The accumulation of CDC45 on chromatin is also negatively correlated with the efficiency of DNA replication in LRR1 knockdown cells. Based on this, the authors conclude that the observed defect in DNA replication is caused by depletion of rate-limiting soluble DNA replication factors. However, the authors do not show that these replication factors, e.g. CDC45, are depleted from the nucleoplasm. To further support their interpretation, the authors should perform a cell fractionation and western blot analysis of CDC45 levels in the cytoplasm, nucleoplasm, and chromatin fractions in G1 phase and in S phase. If all CDC45 is indeed trapped on DNA, there should be a noticeable decrease in CDC45 levels in soluble nucleoplasm in LRR1-deficient cells.
in S phase in comparison to G1 phase and control cells.

We agree that this is a critical experiment. Please see the above section for our detailed description of the subcellular fractionation experiments.

2. Why does LRR1 knockout have a very subtle effect on the EdU incorporation in Figures 2H and S2H in comparison to other experiments?

Thank you for pointing this out. In Figures 2H and S3H of the original manuscript, the EdU incorporation for each siGINS condition was normalized to the matching siCNTL condition. Thus, the siCNTL conditions for sgCNTL and sgLRR1 were normalized to 1, giving the impression that the EdU effects were small. To prevent confusion, in the corresponding Figure 3H of the revised manuscript, we normalized all EdU values to the (sgCNTL + siCNTL) condition, and the effect of LRR1 knockout is now apparent.

3. The authors show that LRR1 loss leads to activation of ATR signaling in G2/M phase. The authors state: "failure to unload CMG helicases after LRR1 loss triggers a G2/M checkpoint that activates the ATR/Chk1/Wee1 pathway, arresting cells in G2 and blocking mitosis". However, the G2 arrest could be a result of failure to modify other Lrr1 targets. This should be discussed.

This is a valid point. We have added a paragraph in the Discussion section to address this possibility. We have also performed an experiment where cells were treated with a p97 inhibitor (CB-5083), which phenocopied LRR1 knockout in blocking mitosis (Figure S5B). This led us to favor our interpretation that the G2 arrest after LRR knockout is due to the failure to unload CMG helicases.

4. In Figure 2A-B, were cells fixed 30 hours after release from serum starvation or 6 hours after release? The legend is not clear on this. 30 hours would be a long time. Irrespective of the time, how do the authors know the cells entered S phase with similar kinetics in the two genotypes?

Thank you for point this out. In Figures 2A-B of the original manuscript, cells were indeed fixed 30 hours after release. We acknowledge that this is not the best time point to compare S-phase phenotypes between control and LRR1 knockout cells. In the revised manuscript, we have replaced these figures with results from asynchronously cycling cells (Figures 2A-B), which avoid the timing issue associated with serum-released cells. We measured cell-cycle progression in these cells (Figure S2B) and found similar percentages of S-phase cells in control and LRR1 knockout cells. To reconcile the fact that (i) percentages of G1/S/G2 cells are not significantly different between control and LRR1-null cells and (ii) there is indeed G2 arrest in these cells (lower percentage of cells that are going into mitosis, as shown in Figure 4), we note that these scatter plots are from cells fixed one day after LRR1 knockout, and this is likely the first cell cycle that the cells went through in the absence of LRR1 protein. As a result, the G2 arrest is just
beginning to manifest and is not captured by a higher percentage of G2 cells.

MINOR COMMENTS:

1. Authors use "normalized to sgCNTL" values for showing quantification of the incorporated EdU signal for Figure 2B, 2H, S2B, S2H, but "R.F.U" values for Figure 2E, S2E, S2G, and S2I. It would be better to use the same units and scales for all these panels for the easiest comparison of these experiments.

We have standardized the units and scales to facilitate comparison and switched to using normalized EdU and CDC45 signal wherever possible.

2. Figure S4G panel is referred in the text prior to panels S4E and S4F, and therefore, the order of panels should be changed accordingly.

Thank you for pointing this out. We have reshuffled the order in the text.

3. Can the authors speculate about the trigger of ATR pathway activation in the absence of LRR1? Do they think that some replication forks eventually stall when neighboring origins fail to fire?

We have added a section in the Discussion to speculate on the trigger of ATR activation based on new data that we acquired. We measured a panel of DNA damage markers, including chromatin-bound RPA1 (single-stranded DNA) and phospho-RPA2-Ser33, in both S-phase and G2-phase cells (Figures S3D, 4I). Interestingly, after LRR1 loss, single-stranded DNA did not accumulate in S-phase cells but in G2 cells. Additionally, DNA fiber assay suggests that fork progression was not significantly affected by loss of LRR1 (Figure 3E-F). These data suggest that fork stalling is not a major issue during S-phase in LRR1-null cells. In addition, we measured mitotic DNA synthesis (Bergoglio et al., 2013; Minocherhomji et al., 2015) and 53BP1 nuclear bodies (Harrigan et al., 2011; Lukas et al., 2011; Spies et al., 2019) to test for under-replicated DNA. We found that while LRR1 depletion resulted in higher DNA synthesis during mitosis, LRR1-depleted cells that went through mitosis did not accumulate chromatin-bound 53BP1 puncta in G1, as opposed to cells pre-treated with aphidicolin (Figure S5L). Our interpretation of these results is that LRR1 depletion results in under-replicated DNA in G2 phase, which is efficiently resolved through mitotic DNA synthesis and not inherited by daughter cells. We speculate that (i) chromatin-bound CMG helicases in G2 actively unwind double-stranded DNA into single-stranded DNA or (ii) single-stranded DNA accumulate in regions with under-replicated DNA. Single-stranded DNA then recruits RPA and triggers ATR activation.
Reviewer #2 (Comments to the Authors (Required)):

In this manuscript, authors examined the role of the E3 ubiquitin ligase CRL2LRR1 in human cells. Authors showed that LRR1-depleted cells accumulate Cdc45 and other replisome components on chromatin and exhibit reduced level of DNA synthesis. They concluded that the accumulation of replisome factors on chromatin due to failure of replisome disassembly inhibits recycling of limited replication factors, thus reducing the level of DNA synthesis. They further show that the slowed/compromised replication (or accumulated replication factors?) activates ATR-Chk1-Wee1 checkpoint, inhibiting M phase progression. Finally they showed that LRR1 is required for proliferation of the mammalian cells.

Generally, the experiments are conducted very carefully. The data presented are convincing and are consistent with the authors' conclusions.

Authors propose that continuous disassembly of replisome, as it terminates during the course of S phase progression, is required for efficient S phase progression. This is an interesting possibility but has not been rigorously examined in the manuscript.

I would like to suggest the authors address following points to clarify some unsolved issues and to further improve the manuscript.

Thank you for the comments. We will address the specific comments below.

Major comments

1. Proteins abundance is examined only by the staining or FACS. The protein level, especially that of Cdc45, needs to be examined by western blotting after cell fractionation. According to the authors' conclusion, the Cdc45 protein level in the detergent soluble fractions after knockout of LRR1 should be reduced. However, this has not been directly shown. The increased staining signals on chromatin most likely reflects the increased level of the protein, but it could be that Cdc45 may be refractory to the detergent extraction, or the antibody epitope may be more accessible due to the change of replisome structure after LRR1 depletion.

We agree that this is a critical experiment, and it is suggested by all three reviewers. We found through subcellular fractionation that LRR1 knockout reduced the levels of CDC45, POLE1, and Timeless in the detergent-soluble pool (Figures 3B-D, S4B-D). LRR1 knockout also increased the levels of CDC45, GINS2, POLE1, and Timeless in the insoluble pool, suggesting that the increased chromatin-bound CDC45 stain in immunofluorescence is not an artifact of epitope masking. Please see our response to the general comment by reviewer 1 for a detailed description of this experiment.
2. Would it also be possible that the only the chromatin-bound Cdc45 is stabilized by LRR1 depletion? This could be examined also by western blotting analyses of the stability of Cdc45 in detergent-soluble and -insoluble fractions.

This is a valid point. To address this point, we performed a stability experiment where we treated cells with a combination of cycloheximide (to block protein synthesis), a CDK1/2 inhibitor (to block origin firing), and a p97 inhibitor (to block CMG unloading), essentially blocking influx and efflux of the detergent-soluble and insoluble pools of CMG components. We treated cells with this cocktail over varying lengths of time, harvested them, and performed subcellular fractionation. We found that the stability of CDC45, GINS2, POLE1, and MCM2 is largely unaffected by LRR1 depletion (Figure R1). Due to space limitation, we did not include this experiment in the supplemental figures of the revised manuscript.

Figure R1. LRR1 was depleted in LRR1−/− cells expressing exogenous, doxycycline-inducible LRR1 by removing doxycycline and transfecting siRNA targeting exogenous LRR1 one day before the experiment. Cells were treated with cycloheximide (100 µg/mL), CDK1/2 inhibitor III (3 µM), and CB-5083 (4 µM) for the indicated amount of time. Band intensity (relative to
GAPDH or H4K20me3 loading controls) was normalized to the 0-hour time point. In the HA-LRR1 blot, the arrow indicates the LRR1-specific band.

3. Although the authors' proposition that the block of the recycling of replication factors inhibits DNA synthesis in LRR1 depleted cells is consistent with the results, it would be also possible that the Cdc45 remaining on the chromatin may be actively inhibiting the process of DNA replication. DNA fiber analyses should be conducted to examine if the fork rate is reduced and/or new origin firing is reduced in LRR1 depleted cells. This will give information regarding how DNA synthesis is inhibited in the absence of LRR1. Related to this, I would also like to see cell cycle distribution of LRR1-depleted cells by simple FACS of PI-stained cells.

Thank you for suggesting this experiment. We have performed DNA fiber assays and found that origin firing was reduced but fork progression was not affected in LRR1-null cells (Figure 3E-F), inconsistent with a model where ongoing forks are stalled by replisomes remaining on chromatin as roadblocks. More generally, we have added a section in the Discussion to expand on alternative interpretations of the data, including the effect of retained replisomes on stalling of active forks, collisions with transcriptional complexes, and the dependence of CMG helicase unloading after lagging strand fork collapse on CRL2LRR1 activity (Vrtis et al., 2021).

We also included an analysis of cell-cycle progression of EdU and Hoechst-stained cells (Figure S2B). The slower EdU incorporation could be seen in the scatter plot. To reconcile the fact that (i) percentages of G1/S/G2 cells are not significantly different between control and LRR1-null cells and (ii) there is indeed G2 arrest in these cells (lower percentage of cells that are going into mitosis, as shown in Figure 4), we note that these scatter plots are from cells fixed one day after LRR1 knockout, and this is likely the first cell cycle that the cells went through in the absence of LRR1 protein. As a result, the G2 arrest is just beginning to manifest and is not captured by a higher percentage of G2 cells.

4 Authors show that LRR1 depletion activates ATR-Chk1-Wee1 checkpoint. Is Chk1 activated (S317 or S345 phosphorylation of Chk1) after LRR1 knockout? Are single-stranded DNA segments accumulated in the absence of LRR1? Does RPA phosphorylation increase? Are DNA damages induced (gamma-H2AX signals)? Examination of these issues would give some insight into what kind of replication stress is generated after LRR1 depletion. (Is it the arrested replication fork, or slowed replication fork, or the incomplete DNA replication [the presence of unreplicated segments] ? ) DNA fiber analyses, suggested in my comment #3, may also give some clue to these issues.

Thank you for suggesting this experiment. We measured a panel of DNA damage markers, including chromatin-bound RPA1 (single-stranded DNA), phospho-RPA2-Ser33, phospho-Chk1-Ser317, and γ-H2A.X, in both S-phase and G2-phase cells (Figures S3D, 4I). Interestingly, after LRR1 loss, single-stranded DNA, phospho-RPA2, and phospho-Chk1 did not accumulate in
S-phase cells but in G2 cells. \(\gamma\)-H2A.X was mildly elevated in both S and G2-phase cells. DNA fiber assay also suggests that fork progression was not significantly affected by loss of LRR1 (Figure 3E-F). These pieces of data, in addition to the inability of checkpoint pathway inhibitors to rescue slowed DNA replication (Figure S3C), suggest that excessive replication stress is not the cause of slowed DNA replication in S phase. However, the presence of chromatin-bound RPA1 in G2 phase suggests that single-stranded DNA is the trigger for ATR activation in G2.

We also measured mitotic DNA synthesis (Bergoglio et al., 2013; Minocherhomji et al., 2015) and 53BP1 nuclear bodies (Harrigan et al., 2011; Lukas et al., 2011; Spies et al., 2019) to test for under-replicated DNA. We found that while LRR1 depletion resulted in higher DNA synthesis during mitosis, LRR1-depleted cells that went through mitosis did not accumulate chromatin-bound 53BP1 puncta in G1, as opposed to cells pre-treated with aphidicolin (Figure S5L). Our interpretation of these results is that LRR1 depletion results in under-replicated DNA in G2 phase, which is efficiently resolved through mitotic DNA synthesis and not inherited by daughter cells.

Thus we speculate that (i) chromatin-bound CMG helicases in G2 actively unwind double-stranded DNA into single-stranded DNA or (ii) single-stranded DNA accumulate in regions with under-replicated DNA. Single-stranded DNA then recruits RPA and triggers ATR activation.

Other comments

5. Authors state that "it is plausible that as a result of increased origin firing and higher demand for replication factors, cancer cells might be more sensitive to reduced levels of rate-limiting replisome components upon loss of CRL2LRR1 activity and sequestration of replisome components on chromatin." This is an interesting possibility but authors only examined one cancer cell line in this manuscript. I would like to know if LRR1 regulates replication termination in non-cancer cells in a manner similar to that operated in cancer cells, since, generally, non-cancer cells have much lower levels of replication factors (including Cdc45 and Cdc7, normally rate-limiting factor for replication initiation, and helicase component Mcm) compared to cancer cells, and may adopt a different strategy to deal with disassembly of replisome. I would appreciate if authors could add any information or discussion regarding this.

This is an interesting point. We note that in the original manuscript, we exclusively used MCF10A cells, which are non-transformed breast epithelial cells. We have expanded the section in the Discussion to speculate on the effect of blocking replisome disassembly in cancer cells.

6. It appears that the EdU pattern in LRR1 depleted cells represent mostly that of mid-S/late-S replicating cells (e.g. Figure 2A). Are these statistically significant? There are also cells that are positive for Cdc45 but are not replicating. In what cell cycle stage are these cells present?
Thank you for pointing this out. In LRR1 knockout cells, DNA replication becomes slower as cells progress through S phase (Figure 2D). As a result, S-phase cells are indeed more enriched for mid/late-S phase cells (see EdU-Hoechst scatter in Figure S2B and Figure R2 for quantification). Cells that are positive for CDC45 but are not incorporating EdU are in G2 phase.

Figure R2. Distribution of DNA content in S-phase cells. Error bars are mean ± standard deviation. Paired Student’s t test, * p < 0.05 (n = 4 independent experiments, n ≥ 4272 cells per condition).

The Cdc45 staining signals are very strong in LRR1 depleted cells. They form rather strong nuclear foci. I wonder if these foci represent the replication termination/or pause sites where Cdc45 needs to be disassembled during normal S phase. I felt it would be interesting to determine by ChIP-seq where Cdc45 is bound on chromatin under this condition. I would not request authors to conduct the experiments, but appreciate if they have any thought on this.

This is an interesting idea. We speculate that these foci represent genomic sites where replisomes terminate, especially in or near replication factories/foci. In the future, combining CDC45 ChIP-Seq and sequencing methods that map termination sites, e.g. Okazaki fragment sequencing (McGuffee et al., 2013; Petryk et al., 2016), may provide insight into how LRR1 knockout affects the distribution of termination sites.
Reviewer #3 (Comments to the Authors (Required)):

In this study by Fan et al, the authors have characterized the replication and proliferation phenotypes of MCF10A cells lacking the LRR1 Cullin adapter. The principle findings are that LRR1-deficient cells retain the CMG helicase on DNA - particularly in late S phase. These cells are also severely compromised for DNA synthesis rate as measured by EdU incorporation during a pulse label. The experiments are highly quantitative image analyses of single cells. There are several strengths of the experimental approach including the analysis of cells acutely depleted of LRR1 within a single cell cycle and the excellent use of a complementing LRR1 construct. The experiments themselves are convincing, and the manuscript is clearly presented.

I have two primary concerns: the significance of the advance and the support for claims and conclusions in the manuscript. I also have some comments on experiments and presentation at the end.

1. Significance. This study is a reasonably thorough characterization of one human cell line, but the unloading defect is expected from several other studies in yeast, worms, and frog oocytes. In this regard, it is confirmation of conservation across species of the role of LRR1 in CMG unloading. The possible novelty here is the correlated defect in the rate of DNA synthesis in human cells which was not noted in the frog model, but is consistent with the S phase accumulation in dia2-null yeast (Blake 2006). Similarly, the transcriptome analysis is well done, but there are a great many datasets already for palbociclib-sensitive genes in many cell lines (perhaps not MCF10A). The focused sgRNA screen is a nice and relatively unbiased approach to finding new cell cycle genes, but not particularly new; it seems one could have started from existing datasets to get to a similar group of 30 genes in S1B.

Thank you for pointing this out. We have performed a set of additional experiments in Figure 3 that support our working model that the slower DNA replication is due to a failure to recycle replisome components into the soluble pool after LRR1 loss. We believe that with these new experiments to establish the mechanism of slowed DNA replication after LRR1 knockout, our manuscript provides insight into the importance of replisome disassembly and recycling, which would benefit the community. We will discuss these experiments in more detail in the following point-by-point response to specific comments.

2. Author Conclusions. There are claims that are not fully supported by the available data: a. In the abstract itself, "cells disassemble active replisomes to prevent re-replication and genome instability." There are no experiments here to address genome instability or re-replication, and the one published experiment in worms suggests but does not directly prove re-replication as the cause of increased DNA content. In fact, the first sentence in the abstract is the only time re-replication appears in the document.

This is a valid point. We have removed this claim from the abstract.
b. Importantly, the authors frequently escalate a correlation they show into a causal relationship. In particular, the fact that LRR1 loss leads to some replication proteins retained on chromatin and to slower DNA synthesis does not automatically mean that they have established retained CMG as the sole or primary cause of the EdU phenotype. LRR1 is a substrate adaptor for an enzyme that almost surely has more substrates than the MCM complex. All of the other substrates would be perturbed in these knockout cell lines, and one or more of them could be the cause of or contribute to a general S phase slowdown. While the model the authors suggest is consistent with their data, they can't claim to have determined the mechanism of DNA synthesis inhibition. To make that claim, they would need to do more than establish the correlation. For example, preventing CMG removal by some other means in LRR1-positive cells should have the same phenotype as the LRR1-negative cells.

This is an excellent point. We have added a section in the Discussion to address alternative mechanisms other than our proposed model. More importantly, to prevent CMG removal by another means, we treated cells with a p97 inhibitor (CB-5083), which phenocopied LRR1 knockout in slowing DNA replication (Figure S2D) and blocking mitosis (Figure S5B). While p97 has a wide range of cellular targets (van den Boom and Meyer, 2018), the fact that we observed similar phenotypes after LRR1 knockout and p97 inhibition led us to favor the interpretation that CMG unloading, a convergent process downstream of both CRL2LRR1 and p97, is responsible for the DNA replication and mitosis phenotypes.

We have also attempted to prevent CMG removal by overexpressing a lysine mutant of MCM7 (MCM7-K28/29R), the homologous mutant of Xenopus MCM7-K27/28R, which has been shown to block MCM7 polyubiquitination and CMG unloading in vitro (Low et al., 2020). Both MCM7-WT and MCM7-K28/29R were properly loaded onto chromatin in G1 and progressively unloaded during S phase (Figure R3A), suggesting that they are both functional. However, MCM7-K28/29R did not block CDC45 unloading and did not affect EdU incorporation (Figures R3B, R3C), suggesting that either (i) K28/29 are not the in vivo polyubiquitination sites or (ii) other lysines could be polyubiquitinated when K28/29 are mutated. This is reminiscent of data in budding yeast, where MCM7 K29 is the only detectable polyubiquitination site in vitro and in vivo, but in vivo other sites can be polyubiquitinated when K29 is mutated (Maric et al., 2017). Thus we were unable to block CMG removal by overexpressing MCM7 point mutants, and we did not include this negative data in the revised manuscript due to space constraint.
Figure R3. HA-tagged MCM7 was driven by a doxycycline-inducible promoter and induced for two days before pre-extraction and fixation. n = 2000 randomly sampled cells are displayed. Data is representative of 2 independent experiments.

c. The authors also claim to have established that the retention of CMG on chromatin sequesters initiation factors that would normally be recycled. Page 6: "Unexpectedly, we show that the failure to unload CMG helicases increasingly slows down DNA replication by sequestering rate-limiting replisome components on chromatin and blocking their recycling."

While it is true that limiting initiation factor recycling is thought to be an important contributor to the replication timing program, there are no experiments here that show the retained components are limiting. The one observation that supposedly supports this notion is Figure 2F.
The authors seem to predict that retaining CMG should lead to "a corresponding increase in the total pool of CDC45..." (page 5, lines 11-13); this implies that they consider LRR1 to promote CMG or Cdc45 degradation counter to other studies of LRR1 orthologs and their own staining data? It is already well known that polyubiquitylation of MCM7 in model systems does not induce degradation, and the experiments here do not show changes in levels dependent on LRR1 in human cells. One might expect an increase in the soluble fraction relative to chromatin, but that was not measured. The fact that there was not much change in levels is not sufficient evidence for a defect in recycling or sequestering limiting components. They attempted to suppress the phenotype by single protein overproduction (Cdc45 or one subunit of GINS) to no effect. In truth, tests of limiting initiation components have typically needed to overproduce four proteins. Therefore, while the sequestering idea is possible, the authors cannot claim to have demonstrated this as the mechanism.

This is a valid point. To clarify, in the original manuscript, we did not consider LRR1 to promote CMG degradation, although our language was unclear and caused the confusion. Since we performed subcellular fractionation that provided direct data on the levels of soluble components, we have removed the data on total CDC45 from the revised manuscript.

We agree that measuring the soluble fraction of replisome components is a critical experiment. We found through subcellular fractionation that LRR1 knockout reduced the levels of CDC45, POLE1, and Timeless in the detergent-soluble pool (Figures 3B-D, S4B-D). Please see our response to the general comment by reviewer 1 for a detailed description of this experiment.

We also acknowledge that single protein overproduction is unlikely to rescue the DNA replication defect, especially given that the levels of multiple replisome components are reduced in the soluble pool after LRR1 knockout. Thus, we took an alternative approach and upregulated the expression of a number of replisome components by de-repressing E2F activity through siRNA knock down of repressive E2Fs (Figure 3G). Please see our response to the general comment by reviewer 1 for a detailed description of this experiment.

Moreover, the authors have not considered alternative explanations beyond testing checkpoint dependence. Retained complexes might affect fork speed or create collisions with replication or transcription complexes, etc.

This is an excellent point. We performed DNA fiber assays and found that LRR1 knockout reduced origin firing but did not affect fork progression (Figure 3F). This result suggests that ongoing forks are not actively stalled by the presence of retained replisomes. We have added a section in the Discussion to expand on alternative interpretations of the data, including the effect of retained replisomes on stalling of active forks, collisions with transcriptional complexes, and the dependence of CMG helicase unloading after lagging strand fork collapse on CRL2LRR1 activity (Vrtis et al., 2021).
All-in-all, these shortfalls mean the current study doesn't actually establish the mechanism of the DNA synthesis defect which is the main novelty. That leaves the documentation of the phenotype the primary advance which seems too thin at this point.

Additional remarks:

i) There is no analysis of MCM7 polyubiquitination which is important to establish that LRR1 is the primary E3 adaptor in these cells.

A recent study published while this manuscript is under review showed that CRL2\textsuperscript{LRR1} is the E3 ligase responsible for MCM7 polyubiquitination in mouse embryonic stem cells (Villa et al., 2021). The fact that LRR1-mediated CMG unloading is highly conserved in animals from worms to mice and humans suggests that LRR1 is very likely to be the primary E3 adaptor in human cells. We have attempted to detect MCM7 polyubiquitination in MCF10A cells by immunoprecipitating CDC45 or MCM7, yet our assays were not sensitive enough to detect endogenous MCM7 polyubiquitination \textit{in vivo}. Indeed, to detect MCM7 polyubiquitination, the authors of the mouse study used a very large number (3–10×10\textsuperscript{8}) of mouse ES cells that are 70% S-phase cells. To achieve a similar number of cells with MCF10A cells, we would need 100–300 confluent 10 cm dishes for each condition, rendering the experiment essentially impossible.

ii) There is no analysis of LRR1 protein in this study. These assays would establish LRR1 protein as a growth/cycle-regulated protein as well as E2F-regulated gene and confirm the knockout success and no alternative Lrr1 protein production.

This is a valid point. We tested multiple commercial antibodies against LRR1 (Abcam 169947, Proteintech 11628-1-AP, Atlas Antibodies HPA069364) in Western blots and successfully blotted for endogenous LRR1 with the Atlas antibody. We validated that LRR1 protein is cell cycle-regulated and CDK4/6 activity-dependent (Figure 1J). We also validated near complete LRR1 depletion in our stable \textit{LRR1}\textsuperscript{−/−} cell lines (Figure S2F).

iii) all experiments are confined to a single cell line with the exception of data mining. It would be improved to test the principle findings in at least one other line.

We knocked out LRR1 in another cell line, non-transformed human retinal pigment epithelial cells (RPE-1 hTERT), and found reduced rate of DNA replication and elevated levels of chromatin-bound CDC45 in S-phase cells (Figure S2C).

iv) What is the fate of LRR1-negative cells that divide in the presence of wee1 inhibitor? Does that completely rescue proliferation or are cells arriving in G1 with under-replicated DNA? If the latter, then unreplicated DNA might be both the activator of the G2/M checkpoint and the source of long-term proliferative failure.
This is an interesting question. We measured mitotic DNA synthesis (Bergoglio et al., 2013; Minocherhomji et al., 2015) and 53BP1 nuclear bodies (Harrigan et al., 2011; Lukas et al., 2011; Spies et al., 2019) to test for under-replicated DNA. We found that while LRR1 depletion resulted in higher DNA synthesis during mitosis, LRR1-depleted cells that went through mitosis did not accumulate chromatin-bound 53BP1 puncta in G1, as opposed to cells pre-treated with aphidicolin (Figure S5L). Similar results were obtained when we quantified the total level of chromatin-bound 53BP1 rather than puncta area. Our interpretation of these results is that LRR1 depletion results in under-replicated DNA in G2 phase, which is efficiently resolved through mitotic DNA synthesis and not inherited by daughter cells. We speculate that under-replicated DNA, in addition to elevated levels of single-stranded DNA in G2 (Figure 4I, chromatin-bound RPA1), may indeed be an activator of the G2/M checkpoint. However, these cells are arrested in G2 and no longer actively cycling, unless forced to bypass the G2/M checkpoint with Wee1 inhibition; this would imply that the long-term proliferative failure is mainly due to G2 arrest.

v) Many bar graphs are data from one single experiment treating each single cell as a replicate. Given JCB’s endorsement of more rigorous comparison of biological replicates (Lord et al JCB 2020), the authors should consider ways to show reproducibility across separate experiments instead of variation within one experiment. The good examples are 2H, 3D etc. Adding lines for the 25th and 75th percentile that look exactly like statistical representations of variation such as SD or SEM is confusing and should be presented differently (e.g. box-and-whiskers).

Thank you for pointing this out. We have replaced all bar graphs treating each single cell as a replicate with biological replicates (independent experiments) throughout the main figures. We have also removed lines that indicate the interquartile range to avoid confusion.

vi) The Discussion is largely a recap of the results and doesn’t add much context or new concepts. Consider a discussion of alternatives, mechanisms, study limitations, what the wee1 pathway is activated by, etc.

This is an excellent point. We have substantially expanded the Discussion to provide context, alternative interpretations, etc.
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April 27, 2021

RE: JCB Manuscript #202009147R

Prof. Tobias Meyer
Stanford University
Department of Chemical and Systems Biology Stanford University 318 Campus Drive
Clark Building W200
Stanford, CA 94305

Dear Prof. Meyer,

Thank you for submitting your revised manuscript entitled "LRR1-mediated replisome disassembly promotes DNA replication by recycling replisome components". The reviewers find that the revisions strengthened the study and addressed the issues previously raised in review. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below). Please also consider the final remaining points from the referees as you prepare your final files.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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citation "Dempster, J.M., J. Rossen, M. Kazachkova, J. Pan, G. Kugener, D.E. Root, and A. Tsherniak. 2019. Extracting Biological Insights from the Project Achilles Genome-Scale CRISPR Screens in Cancer Cell Lines. bioRxiv:720243."

5) A summary paragraph of all supplemental material should appear at the end of the Materials and methods section.
- Please include one brief descriptive sentence per item.

6) Author contributions: A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Sincerely,

David Gilbert, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Overall, the authors have responded effectively to the points raised by all three reviewers, and the paper is essentially ready for publication.

Minor points:

1. First sentence of abstract is incorrect. Replisomes are unloaded when converging forks meet, not when the entirety of DNA replication is finished.

2. Typo: Line 31 should read "fork protection complex"

3. The legend of Figure 3C does not state the relative amounts of Soluble and Insoluble proteins loaded so it is impossible to verify the authors' conclusion that a small fraction of total protein was in the insoluble fraction.

Reviewer #2 (Comments to the Authors (Required)):

Authors have revised the manuscript with additional experiments, and responded to my comments in a satisfactory manner. Accordingly, the revised manuscript has been significantly improved and now I recommend the publication of this manuscript in Journal of Cell Biology.

Reviewer #3 (Comments to the Authors (Required)):

I am satisfied with these additions and revisions. The new model in Figure 6 is generally helpful. The effect on soluble replication proteins is modest, but detectable. The inclusion of E2F manipulation and p97 inhibition shores up the argument however.