JCB

Intrinsic checkpoint deficiency during cell cycle re-entry from quiescence

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Re: JCB manuscript #201902143

Prof. Jeanette Gowen Cook
University of North Carolina at Chapel Hill
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Dear Prof. Cook,

Thank you for submitting your manuscript entitled "Intrinsic checkpoint deficiency during cell cycle re-entry from quiescence". We sincerely apologize for the delay in communicating our decision to you. The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers were overall positive about the manuscript. They, and we agree, found the work of high quality and feel that it will provide an important and exciting advance for the field. Most of their points seem relatively straightforward to address in the text to provide clarifications and additional information/discussion. We encourage you to address all their points to the best of your ability. In particular, it would seem important to us to respond to the reviewers about their questions around the concept of checkpoint. We realize that Rev#1 point #19 might not appreciate that inhibiting MCM loading is sufficient to lengthen G1 phase, but that needs to be made clear, perhaps with some more explanation in the main figure or legend. It also seems that the ref may not appreciate that G1 is extended when Cdt1 is knocked down (point #10). Despite this confusion, and the confusion in points #20-21 that seem somewhat unlikely because licensing cannot occur after entry into S phase, this is valuable feedback because the model, interpretation, and foundational knowledge on which they are based need to be clarified for a broad readership. Since the checkpoint is poorly defined mechanistically, it is important to make clear why the reviewer's model may not be correct. It is still formally possible that there is something indirect going on, so clearly explaining why a licensing checkpoint is the simplest interpretation will go a long way towards increasing the impact of the paper, which we feel has the potential to bring this poorly understood checkpoint to the forefront of the field. Most of the remainder of the points are simpler clarifications. Finally, we would like to see you fully address Rev#1 point #5, as we were also confused about this quantification.

In summary, please keep in mind that the paper should be accessible to a broad audience of diverse cell biologists, so it seems to us that some of Rev#1's questions offer the opportunity to clarify the concepts you are proposing and to provide more background information to support your interpretations and conclusions. Please let us know if you have any questions about the reviewers' points or anticipate any issues addressing them. We would be happy to discuss the revisions further as needed.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal

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Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

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)):

The authors investigate the loading of the MCM complex onto DNA and compare the first cell cycle from quiescence to subsequent cell cycles. They find that DNA is "underlicensed" in the first cell

cycle from quiescence, that is, there are fewer MCM proteins loaded, in comparison to subsequent cycles. The authors find that this under-licensing makes the first cycle from quiescence particularly prone to DNA damage. The authors then show that knocking down Cdt1 results in a longer G1 that can be overridden by cyclin E overexpression. They show that the effects of Cdt1 knockdown on cell cycle requires p53. Further, p53 inactivation affects cell cycle for subsequent cycles but not the first cycle from G0. Finally, by extending the first cell cycle with p53 stabilization, the first cycle can resemble subsequent cycles, while overexpressing cyclin E can make the 2nd cell cycle resemble the first in terms of MCM loading.

The topic will be of interest to those in the field. The data are overall relatively convincing and mostly strong. The manuscript is extremely well-written and clear. There are some cases in which the data and the plots that summarize the data do not seem to match. Further, the authors argue forcefully for a "checkpoint," but other explanations for the data may also be possible. Nevertheless, overall the paper is an important and exciting advance for the field and should be suitable for publication after the authors address the comments below. The most critical issues are identified with asterisks.

1. Figure 1G. It would be helpful to know what happens with serum starvation in the fibroblasts.

2. It is confusing that there is primary data in Fig 1C, then a western in Fig 1D, then the data in Fig 1C is presented again in a different way in Fig 1E and Fig 1F. Can the authors please reorganize Fig 1 so that all of the representations of the data in Fig 1C are together?

3. Fig 1A provides helpful information on MDM loading, but the fact that it is pre-gated on G1 S and G2/M based on Click iT is in the supplement, and the reader may not understand based on what's in Fig 1A. Moving the third plot in Fig S1 to the main text would likely make clear how the Click-iT data is being used to generate this figure.

4. It would be helpful to see the Click iT edU traces for the data in Fig 1C for 1st cell cycle and 2nd cell cycle. In particular, the early S phase in the box in Fig 1E represents cells that are located in what position on the Click iT edU plot? They have 2N DNA content but are EdU positive? How positive? Is it similar for the 1st vs 2nd cell cycle?

5. *Fig 1G: the plot of 1st vs 2nd cycle for serum starvation looks a little surprising. The primary data in the supplement give the impression that the difference is significantly more subtle for serum starvation than contact inhibition, but the summary data in Fig 1G don't seem to agree.

6. Why is mid S phase investigated in Fig 2 and very early S phase investigated in Fig 1? The switch may be confusing for readers and should be carefully explained. For instance, if the biggest difference in MCM loading is at the G1/S boundary, why not look for differences in gemcitabine there rather than later in S phase?

7. Why is it necessary to treat with gemcitabine to detect a difference in replication stress? If the cells are so underlicensed in the first cell cycle from quiescence, why don't they show a difference in the fraction of cells with high gamma-H2Ax compared with controls even without gemcitabine?

8. It seems that once the cells start cycling, there is a reset and MCM loading is as usual. So what is the reason that the cells would be more sensitive to gemcitabine with multiple rounds of contact inhibition and release? Wouldn't any issues with low licensing three contact inhibitions ago be addressed by that cell at the time (either it replicates ok anyway, or it has to pause for replication,

or it gets some sort of mutation or larger DNA damage) rather than causing some sort of lingering effect? Could it be that they have been through more passages and are older (more time in culture, more passages?) rather than an effect from previous contact inhibited periods? There are reports of memories of dormant origin use (Blow, Coubert), are the authors expecting that this is contributing?

9. The authors provide information on gemcitabine sensitivity from multiple rounds of contact inhibition and release, but not information on MCM loading. Is MCM loading different in the cells if they have been contact inhibited previously?

10. *The authors are arguing in Fig S3A, B that there is a change in MCM loading in G1 with cdt1 knockdown. The earlier studies focused on early S. I cannot actually see this change from the FACS plots and am not sure how meaningful MCM loading in G1 is as these cells are likely a heterogeneous mixture of cells with 2N DNA content. The data look very similar for WT vs KO p53, and here the authors are arguing that the absence of p53 had little effect on G1 phase MCM loading while similar data are interpreted as showing significant changes for cdt1 knockdown.

11. *The claim is that cyclin E overexpression combined with an siRNA against Cdt1 leads to dramatic underlicensing. When I look at Figure S3A, I don't see this effect. In particular, in Fig 3D, if I look with and without cyclin E1 overexpression, the effect of the siCdt1 B knockdown looks minimal. I don't understand how the data in Fig 3D for siCdt1B could have been derived from the plot in Fig 3C. The siCdt1/siControl ratio looks similar for cyclin E1 overexpression. I don't think that number should be 50%. Fig 3E looks correct. The fraction underlicensed looks similar with cylin E1 overexpression whether or not Cdt1 is knocked down.

12. The authors argue that there must be a bona fide checkpoint since cyclin E overexpression results in shorter G1 with siCdt1 knockdown. Some questions arise: 1. Cyclin E1 overexpression leads to shorter G1 in cells with control siRNA. Are they checkpoint arrested? 2. What if there's a competition between levels of cdt1 and cyclin E and the G1 length is a result of this competition. Would it not look the same as the data shown? Would that be a real checkpoint?

13. The western in Fig 4B is smeary in a way that makes it hard to interpret what the correct levels of p53 and p21 are in the control p53WT lane.

14. *When I look at Figure S3C, I don't see that 70% of the cells are underlicensed in the p53 KO siCdt1B sample. siCdt1A knockdown looks more convincing. But Cdt1 B siRNA had been stronger than A in Fig 3F.

15. *In Fig 5B, how was this experiment performed? This is a snapshot of one timepoint for cells in a first cell cycle? When are the cells collected? By the time this snapshot was taken for flow cytometry, the cells are actively dividing. What happened before this timepoint? Same for Fig 5D? In general, the expectation is that the first cycle has a longer G1 than later cycles, so these results may be at a late and less relevant timepoint.

16. Overall, the results are confusing: why would p53 know that this is the first cycle and not perform its usual function of acting as a checkpoint?

17. In Fig 6A, can the authors please show the same times that are highlighted with circles on the plot in Fig 6B?

18. What is the meaning of dividing the licensing window time by Cdc6 peak time? Is the licensing window time determined by Cdc6 levels?

19. *Is it necessarily a lack of a "checkpoint"? Indeed, increasing p53 and giving the cells more time for MCM to be recruited can make the first cycle more like a second, while increasing cyclin E and thereby reducing the available time for licensing, can make the second cycle more like the first according to the data in Fig 7. This seems like a model involving a competition between loading and time available. But the idea that there is something sensing whether MCM is loaded or not, and preventing the cell from progressing if it is not, has not been shown. The authors have elucidated a model for loading and S phase entry with molecular detail; it's just a question of whether a "checkpoint" is the most accurate description.

20. *Is it possible that p53 is induced by DNA damage that occurs when a cell does go into S phase with too few MCM complexes loaded and then it activates p21 which slows down the cell cycle and allows for more loading. Then there is a checkpoint but it is a DNA damage checkpoint, not a loading checkpoint.

21. *Similarly, the argument is that if cyclin E overrides the effect, then it is a checkpoint. What if cyclin E shortens the cell cycle? It seems that if just overexpressing cyclin E is sufficient to make a second cycle look like a first, even without inactivating p53, then that argues that it's there isn't a surveillance mechanism. Maybe it just shows that cells will go into the cell cycle with whatever MCM loading they have at the time they commit.

22. Finally, the authors don't monitor total MCM levels. Maybe for the first cycle, the cells don't have as much MCM to load, so when they enter the cell cycle, some cells are not well licensed?

Formatting issue

1. Figure 1 is printing with the left side cutoff.

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

Matson et al have investigated controls over the licensing of replication origins in proliferating cells and in 'normal' RPE1 cells re-entering the cell cycle from G0. They show, surprisingly, that some cells re-entering the cycle from G0 have significantly reduced quantities of DNA-bound MCM2, indicative of a reduced number of licensed replication origins. Cells entering S phase from G0 also had an increased amount of γH2AX, consistent with replication problems arising from a reduction in the number of total potential origins. The underlicensing was in part due to a defective p53-dependent licensing checkpoint which was specifically defective in cells re-entering the cell cycle from G0. This is a very nice paper which draws an important new conclusion about the licensing checkpoint and showing that its loss creates a unique vulnerability in cells re-entering the cell cycle. The data are very clear, and I think the paper is suitable for publication as it stands, though I have several additional questions. It would be great to know why MCM loading is slow on G0 exit: the obvious answer would be to do that Cdt1 levels are lower than in cycling cells - have the authors looked to see if this is the case? It would also be very interesting to know what is different about the licensing checkpoint on cell cycle re-entry, but I guess that is all for another paper.

Minor Point

Fig 2B - what threshold was applied to distinguish γH2AX-positive cells? Could the cells be gated for MCM levels to show that the underlicensed cells are the ones that have increased γH2AX?

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

Cell license origins in G1, forming an excess of origins that are crucial for situations in which replication stress is encountered. A key step in origin licensing involves MCM loading, which occurs in G1. Given the critical nature of this process, proliferating cells can delay entry into S phase if licensing is not complete through activation of a p53-dependent checkpoint. G1 lengths can vary in different cell types and previous work from this group showed that stem cells, which have a very short G1, are able to more rapidly load MCMs. In fact, these cells ultimately achieve the same loading of MCMs upon S phase entry.

In this paper, the loading of MCMs is examined in cells that exit quiescence or G0. During the time from G0 exit to S phase entry, origin licensing and MCM loading must occur and because the time in G1 is considerably longer in this transition relative to a proliferating cell, it is hypothesized that origin licensing may be different. Surprisingly, the authors find that instead of loading more MCMs with the additional time available in the first G1 after quiescence, cells leaving G0 load less MCMs. Accordingly, these cells are more sensitive to replication stress, as monitored by measurement of H2AX phosphorylation. Additionally, the authors find that the origin licensing checkpoint in the first G1 after quiescence is compromised relative to that seen in the next cell cycle and that MCM loading proceeds more slowly.

This is an interesting and careful study that takes advantage of quantitative flow cytometry and single cell imaging to carefully monitor MCM loading, G1 and S phase progression and DNA damage in a normal human fibroblast cell line. The authors nicely and quantitatively demonstrate a difference in MCM loading in the first vs second cell cycle and go on to convincingly show this is due to slower MCM loading and a defect in a bona-fide checkpoint. Thus there is a naturally occurring scenario in which origins are under-licensed. Although the observation itself is surprising, it could help explain a number of observations about hematopoietic stem cells. It also suggests that repeated rounds of quiescence and cell cycle reentry - as induced by some virus and drug treatments, could lead to accumulated DNA damage in cells over time. The studies are all very carefully controlled and the data are well presented. Furthermore, the authors conclusions are well justified by the data shown. I think the work would be of general interest to the readership of J Cell Biology. Nevertheless, I have some minor comments that should be addressed before publication.

Figure 3C - why is there no further decrease in MCM loading or the percentage of underloaded cells when Cdt1 is knocked down together with cyclin E expression (vs knockdown alone). I would hypothesize that these would have an additive effect.

Figure 3F - why is the G1 phase still longer with knockdown of Cdt1 and cyclin E overexpression? Does this suggest there is some partial checkpoint is in place?

Figure 4F - why do the authors think that Cdt1A and Cdt1B siRNAs have different effects on the G1 population in the p53 KO cells if the checkpoint is lost? Shouldn't they both look similar to the KO with siControl?

Dear Drs. Gilbert and Casadio,

We are very pleased at the positive comments from all the reviewers and in your editorial response. Each point is addressed below. Please note some figure numbering has changed, and we have pointed to these changes in our responses. New data included in revision are in: Fig 1G, H, Fig 2Ei-Giii, Fig S1B, C, E, H, I, Fig S2E-K, Fig S3B, F-K. New text in the manuscript that responds to reviewer critiques is highlighted in blue, and we have made revisions to conform to JCB length requirements. We are optimistic that this improved version of the study satisfies the requirements for publication in JCB and look forward to your decision. Thank you to the reviewers and editors for the time and thoughtful consideration.

Jean Cook, PhD and co-authors

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

1. Figure 1G. It would be helpful to know what happens with serum starvation in the fibroblasts.
We have added serum starvation and re-stimulation of NHF1 and Wi38 in Fig 1G, H, Fig S1H, I, Fig S2E, F. These cells are underlicensed to a similar degree as when released from contact inhibition.

2. It is confusing that there is primary data in Fig 1C, then a western in Fig 1D, then the data in Fig 1C is presented again in a different way in Fig 1E and Fig 1F. Can the authors please reorganize Fig 1 so that all of the representations of the data in Fig 1C are together?
We rearranged Fig 1, now the western is Fig 1C, and the flow cytometry Fig 1D.

3. Fig 1A provides helpful information on MDM loading, but the fact that it is pre-gated on G1 S and G2/M based on Click iT is in the supplement, and the reader may not understand based on what's in Fig 1A. Moving the third plot in Fig S1 to the main text would likely make clear how the Click-iT data is being used to generate this figure.
The gating in Fig S1A is arranged together to show the hierarchical gating scheme from raw data on the left to analyzed cell populations on the right. Separating the gates would make the hierarchy harder to follow.

4. It would be helpful to see the Click iT edU traces for the data in Fig 1C for 1st cell cycle and 2nd cell cycle. In particular, the early S phase in the box in Fig 1E represents cells that are located in what position on the Click iT edU plot? They have 2N DNA content but are EdU positive? How positive? Is it similar for the 1st vs 2nd cell cycle?
We added the first and second cycle EdU+DAPI plots as Fig S1E for data shown in Fig 1D (previously Fig 1C). We marked the early S phase cells analyzed in Fig. 1E orange; they are similar in first and second cell cycles.

5. *Fig 1G: the plot of 1st vs 2nd cycle for serum starvation looks a little surprising. The primary data in the supplement give the impression that the difference is significantly more subtle for serum starvation than contact inhibition, but the summary data in Fig 1G don't seem to agree.
The reviewer is correct in that the appearance of the representative data chosen for Fig S2B (previous Fig S1J) gives the impression that the underlicensing was less severe for serum-starved vs contact-inhibited. However, the average in Fig. 1G are the means of multiple replicates. Fig 1G is a ratio of mean loaded MCM, and the values for the particular replicate shown in Fig S2B are 2469 (first cycle mean early S loaded MCM) divided by 3961 (second cycle mean early S loaded MCM) = 0.62. Moreover, we note that the *shape* of the histograms can easily lead a reader to focus on the relative positions of the peaks at the expense of

comparing the full distribution of the data. Note for instance that the maximum MCM loading for the 1st cell cycle (orange lines) is quite lower than for the 2nd cell cycle (grey lines), and some populations have larger or smaller tails.

6. Why is mid S phase investigated in Fig 2 and very early S phase investigated in Fig 1? The switch may be confusing for readers and should be carefully explained. For instance, if the biggest difference in MCM loading is at the G1/S boundary, why not look for differences in gemcitabine there rather than later in S phase?
In Fig 1 we analyzed very early S phase to measure the amount of MCM that had been loaded in G1 at the time cells start S phase before any substantial MCM unloading from replication fork termination. We measured mid-S for DNA damage in Fig. 2 because replication stress-induced damage would be a consequence throughout S phase and especially in when replication is most active in mid-S. We made text changes on pages 6 and 7 to clarify the selection of these populations for analysis.

7. Why is it necessary to treat with gemcitabine to detect a difference in replication stress? If the cells are so underlicensed in the first cell cycle from quiescence, why don't they show a difference in the fraction of cells with high gamma-H2Ax compared with controls even without gemcitabine?
After a ~2 fold reduction in loaded MCM during the first cycle endogenous replication stress is low. This observation is consistent with other studies to test the consequences of reduced MCM loading, and those studies also relied on drug-sensitivities to detect a difference in replication stress. Ge et al 2007 also used drug treatment (HU) to show difference in gamma-H2AX after ~50% reduction in chromatin loaded MCM. We chose gemcitabine because, like HU, it perturbs nucleotide pools and it is also a common chemotherapeutic drug in current use. To further bolster this section of the study, we have also added new data in Fig 2 staining for DNA-loaded RPA and treatment with etoposide, another common chemotherapeutic. These results are fully consistent with the result measuring g-H2AX after gemcitabine treatment. These data are Fig. 2Ei-Giii, Fig S3B.

8. It seems that once the cells start cycling, there is a reset and MCM loading is as usual. So what is the reason that the cells would be more sensitive to gemcitabine with multiple rounds of contact inhibition and release? Wouldn't any issues with low licensing three contact inhibitions ago be addressed by that cell at the time (either it replicates ok anyway, or it has to pause for replication, or it gets some sort of mutation or larger DNA damage) rather than causing some sort of lingering effect? Could it be that they have been through more passages and are older (more time in culture, more passages?) rather than an effect from previous contact inhibited periods? There are reports of memories of dormant origin use (Blow, Coubert), are the authors expecting that this is contributing?
We added new data in Fig S3F and S3G with proliferating cells 8 passages apart (24 days), showing no difference in drug sensitivity. Moreno et al 2016 previously showed a 50% reduction in loaded MCM causes unreplicated DNA to pass through mitosis, we suspect some similar damage builds up over time with repeated underlicensing. However we cannot rule out other effects, such as origin memory from altered replication timing, ROS accumulation, altered DNA repair in G0 or other stress effects caused by G0 itself instead of changes in MCM loading specifically. We added text on page 15 to clarify.

9. The authors provide information on gemcitabine sensitivity from multiple rounds of contact inhibition and release, but not information on MCM loading. Is MCM loading different in the cells if they have been contact inhibited previously?

We added new data in Fig S3H-K showing that the amount of underlicensing in the first cell cycle does not significantly change between 1xG0 and 3xG0.

10. *The authors are arguing in Fig S3A, B that there is a change in MCM loading in G1 with cdt1 knockdown. The earlier studies focused on early S. I cannot actually see this change from the FACS plots and am not sure how meaningful MCM loading in G1 is as these cells are likely a heterogeneous mixture of cells with 2N DNA content.
The data look very similar for WT vs KO p53, and here the authors are arguing that the absence of p53 had little effect on G1 phase MCM loading while similar data are interpreted as showing significant changes for cdt1 knockdown.
The change in G1 MCM loading is easiest to notice in the histogram of the current Fig S4B (previously Fig S3B), where the blue and green lines are clearly shifted from the black control line to lower values of loaded MCM. This decrease is also visible on the dot plots in Fig S4A (previously Fig. 3SA) as the abundance of more blue and grey cells with 2C DNA content, low on the y-axis (loaded MCM), but it is easier to see on the histogram. In contrast, the leftmost histogram of Fig. S4D (previously S3D), p53 WT vs KO siControl the two lines nearly overlap, indicating no difference in G1 phase MCM loading. We added text on pages 8 and 9 for emphasis on the expected MCM loading defect from Cdt1 depletion.

11. *The claim is that cyclin E overexpression combined with an siRNA against Cdt1 leads to dramatic underlicensing. When I look at Figure S3A, I don't see this effect. In particular, in Fig 3D, if I look with and without cyclin E1 overexpression, the effect of the siCdt1 B knockdown looks minimal. I don't understand how the data in Fig 3D for siCdt1B could have been derived from the plot in Fig 3C. The siCdt1/siControl ratio looks similar for cyclin E1 overexpression. I don't think that number should be 50%. Fig 3E looks correct. The fraction underlicensed looks similar with cylin E1 overexpression whether or not Cdt1 is knocked down.
Thank you for pointing out this omission; we should have highlighted these differences earlier; Reviewer #3 had a similar question. There are more underlicensed cells with siCdt1+Cyclin E, but the change is primarily in the number of cells below our MCM antibody threshold. They score as MCM negative and are not included in the early S histogram, but are visible in Fig S4A (previously S3A) as a population of grey MCM^{DNA} neg. cells in S phase. Because we cannot formally distinguish cells that are MCM-negative for technical reasons (e.g. extraction, staining) from those that are truly biologically MCM-negative, we have not included them in our analyses. We added text on page 9 to make these points. Additionally, we found that our original figure legends in Fig 3D, and Fig 4D were unclear and have changed the legends.

12. The authors argue that there must be a bona fide checkpoint since cyclin E overexpression results in shorter G1 with siCdt1 knockdown. Some questions arise: 1. Cyclin E1 overexpression leads to shorter G1 in cells with control siRNA. Are they checkpoint arrested? 2. What if there's a competition between levels of cdt1 and cyclin E and the G1 length is a result of this competition. Would it not look the same as the data shown? Would that be a real checkpoint?
1. The cells are not arrested.
2. The timing of S phase entry is dependent on CDK2 activation. To our knowledge, neither Cdt1 nor MCM loading controls CDK2 activity directly like Cyclin E does, see Fig 3A diagram. The established effects of inhibiting MCM loading on G1 length require a relationship between MCM loading and CDK activity that is very unlikely to be biochemically direct binding competition. We consider a checkpoint control linking MCM status to CDK activity the most likely explanation for the observation and have revised the text on page 8 to clarify the evidence for the checkpoint's existence.

13. The western in Fig 4B is smeary in a way that makes it hard to interpret what the correct levels of p53 and p21 are in the control p53WT lane.
We re-ran the same sample on a new gel and replaced the western with a new image in Fig 4B. We added text on page 9 to aid interpretation of the p53 and p21 changes.

14. *When I look at Figure S3C, I don't see that 70% of the cells are underlicensed in the p53 KO siCdt1B sample. siCdt1A knockdown looks more convincing. But Cdt1 B siRNA had been stronger than A in Fig 3F.
For the dot plots of p53 KO siCdt1 B, note the y-axis height is substantially lower compared to p53 WT siControl in Fig S4C (previously S3C). The easiest way to visualize the difference is the histograms, which more clearly show the density distribution of early S phase cells in Fig 4C, compare the black lines to the orange lines.

15. *In Fig 5B, how was this experiment performed? This is a snapshot of one timepoint for cells in a first cell cycle? When are the cells collected? By the time this snapshot was taken for flow cytometry, the cells are actively dividing. What happened before this timepoint? Same for Fig 5D? In general, the expectation is that the first cycle has a longer G1 than later cycles, so these results may be at a late and less relevant timepoint.
This experiment is indeed a single timepoint, where G0 cells were incubated with siRNA upon release into the first cycle and collected 24 hours after release from G0, when few cells have yet divided. We also refer the reviewer to new data in Fig. S2G for additional timepoints from the first cell cycle in control cells. We added text on page 10 to clarify.

For the proliferating samples, unsynchronized cells were collected after 72 hours of siRNA treatment. These were included to re-emphasize the proliferating RPE1 normally do have a functioning checkpoint for comparison.

16. Overall, the results are confusing: why would p53 know that this is the first cycle and not perform its usual function of acting as a checkpoint?
We are also interested in a molecular explanation for strong checkpoint activity in proliferating cells but weak activity in the first cell cycle. Achieving that explanation is outside the scope of this work because the molecular pathway connecting origin licensing to S phase entry mediated by p53 is still unknown; it is a topic for ongoing projects however. We also refer the reviewer to the recent study from the Meyer lab that observed similar unusual checkpoint activity in the first cell cycle relative to subsequent cycles (Daigh et al 2018).

17. In Fig 6A, can the authors please show the same times that are highlighted with circles on the plot in Fig 6B?
We have improved Figure 6 by adding labels for the circled time points in Fig 6A. In Fig 6B, we also changed the Cdc6 nuclear intensity trace to indicate nuclear envelope breakdown.

18. What is the meaning of dividing the licensing window time by Cdc6 peak time? Is the licensing window time determined by Cdc6 levels?
We thank the reviewer for pointing out that this comparison is not helpful, and we have removed it.

19. *Is it necessarily a lack of a "checkpoint"? Indeed, increasing p53 and giving the cells more time for MCM to be recruited can make the first cycle more like a second, while increasing cyclin E and thereby reducing the available time for licensing, can make the second cycle more like the first according to the data in Fig 7. This seems like a model involving a competition between

loading and time available. But the idea that there is something sensing whether MCM is loaded or not, and preventing the cell from progressing if it is not, has not been shown. The authors have elucidated a model for loading and S phase entry with molecular detail; it's just a question of whether a "checkpoint" is the most accurate description.
Despite the fact that the molecular basis of this relationship is not yet fully understood, we maintain that the relationship between origin licensing status and S phase entry bears the hallmarks of a cell cycle checkpoint. We added text on page 8 to explain this interpretation. In addition, we have also now explicitly pointed out on page 10 that p53 loss – unlike Cyclin E overproduction - did not shorten G1 but it did eliminate checkpoint behavior. If it were simply a matter of hours in G1, then p53 loss should not have allowed underlicensing in cells with the same G1 length as WT. In the p53 null cells, Cdt1 depletion did not lengthen G1 phase and cells entered S underlicensed whereas WT cells delayed in G1 and were fully licensed in S. (Fig 4).

20. *Is it possible that p53 is induced by DNA damage that occurs when a cell does go into S phase with too few MCM complexes loaded and then it activates p21 which slows down the cell cycle and allows for more loading. Then there is a checkpoint but it is a DNA damage checkpoint, not a loading checkpoint.
There is no MCM loading in S phase, it is prevented by Cdt1 degradation, Orc1 degradation, Cdc6 nuclear export, and expression of the Cdt1 inhibitor Geminin. Because these are asynchronous cells, it is possible that underlicensed cells in S phase accumulate DNA damage then induces p21 in the following G2. However, this is separate from the G1 extension due to licensing checkpoint, and bypass of the licensing checkpoint by Cyclin E/ p53 KO occurs before underlicensing and subsequent DNA damage. Additionally, in Liu et at 2009 and Nevis et al 2009, synchronized cell experiments show the origin licensing checkpoint functioning before any cells enter S phase and accumulate damage.

21. *Similarly, the argument is that if cyclin E overrides the effect, then it is a checkpoint. What if cyclin E shortens the cell cycle? It seems that if just overexpressing cyclin E is sufficient to make a second cycle look like a first, even without inactivating p53, then that argues that it's there isn't a surveillance mechanism. Maybe it just shows that cells will go into the cell cycle with whatever MCM loading they have at the time they commit.
If the reviewer's model is correct – that MCM loading is not coupled to S phase entry and the amount of licensing is a passive product of time in G1 – then WT cells in which MCM loading has been inhibited should start S phase "on time" and be underlicensed. This is clearly not the case because WT cells lengthen G1 when licensing in inhibited (shown here and in previous studies from us and others). We argue that Cyclin E overproduction bypasses the checkpoint because cells enter S underlicensed, but it is also true that overproduction greatly shortens G1 phase. The underlicensing of Cyclin E-overproducing cells is consistent with a checkpoint bypass, but it is not the only evidence for the checkpoint's existence in WT cells. We have added text to page 8 to better explain our conclusion.

22. Finally, the authors don't monitor total MCM levels. Maybe for the first cycle, the cells don't have as much MCM to load, so when they enter the cell cycle, some cells are not well licensed?
We monitored total MCM levels by western blot in Fig 1C (previously Fig 1D), and the cells have the same amount of total MCM in both conditions. Moreover, it has been established in multiple systems that the majority of MCM complexes remain soluble in G1, and moderate changes in total MCM have little effect on loading (Siddiqui et al 2013, Todorov et al 1995).

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

It would be great to know why MCM loading is slow on G0 exit: the obvious answer would be to do that Cdt1 levels are lower than in cycling cells - have the authors looked to see if this is the case?
We agree that it would be exciting to learn why MCM loading is slow. We also considered Cdt1 or Cdc6 as an explanation. We directly overexpressed Cdt1 and stable Cdc6 together in the 1st cell cycle, but this did not prevent underlicensing, new Fig S5B-F, suggesting any difference in Cdc6 or Cdt1 do not fully explain the slow licensing. We added text on page 13 in the Discussion to point out this observation. It may be there is an unknown inhibitor of MCM loading during cell cycle re-entry that was needed in G0 cells to prevent loading in G0, or an unknown activator that is missing during cell cycle re-entry. Identifying such mechanisms is a topic for future study.

Minor Point
Fig 2B - what threshold was applied to distinguish γH2AX-positive cells? Could the cells be gated for MCM levels to show that the underlicensed cells are the ones that have increased γH2AX?
Any cells with γH2AX levels greater than the basal γH2AX levels in the top 5% of in untreated cells are scored positive (Fig 2C). We clarified the text on page 7.
A correlation between the degree of underlicensing and γH2AX would indeed be an interesting one to establish. We found little correlation between loaded MCM levels and γH2AX in individual cells. In general however, cells in the 2nd cycle have more MCM loaded and less γH2AX, and cells in the 1st cycle have less MCM loaded and more γH2AX.

Reviewer #3:

Figure 3C - why is there no further decrease in MCM loading or the percentage of underloaded cells when Cdt1 is knocked down together with cyclin E expression (vs knockdown alone). I would hypothesize that these would have an additive effect.
We thank the reviewer or pointing out this omission in our text; reviewer 1 asked a related question (point # 11). There are more underlicensed cells with siCdt1+Cyclin E, but the change is primarily in the number of cells below our MCM antibody threshold. They score as MCM negative and are not included in the early S histogram, but are visible in Fig S4A (previously S3A) as a population of grey MCM^{DNA} neg. cells in S phase. Because we cannot formally distinguish cells that are MCM-negative for technical reasons (e.g. extraction, staining) from those that are truly biologically MCM-negative, we have not included them in our analyses. We added text on page 9 to make these points.

Figure 3F - why is the G1 phase still longer with knockdown of Cdt1 and cyclin E overexpression? Does this suggest there is some partial checkpoint is in place?
The reviewer may be correct that cyclin E overproduction does not fully bypass the effects of Cdt1 depletion with respect to G1 length. We are mindful however that the inducible Cyclin E cell line is polyclonal, and perhaps only some of the cells express enough Cyclin E to overcome the checkpoint. A separate ongoing study using a monoclonal line responds with a stronger and more uniform G1 shortening. We added text on page 9 and in the materials and methods page 16 to clarify that the cell line used here is polyclonal.

Figure 4F - why do the authors think that Cdt1A and Cdt1B siRNAs have different effects on the G1 population in the p53 KO cells if the checkpoint is lost? Shouldn't they both look similar to the KO with siControl?

We noted this difference also, but we don’t know precisely why the siRNAs have different effects on this particular parameter. SiCdt1 A is a pool of 4 oligo sequences, and one or more of those 4 might have off-target effects that are only revealed in p53-null cells.

May 14, 2019

RE: JCB Manuscript #201902143R

Prof. Jeanette Gowen Cook
University of North Carolina at Chapel Hill
Department of Biochemistry & Biophysics, Campus Box 7260 120 Mason Farm Rd.
Chapel Hill, NC 27599-7260

Dear Prof. Cook,

Thank you for submitting your revised manuscript entitled "Intrinsic checkpoint deficiency during cell cycle re-entry from quiescence". Thank you for your patience as we editorially assessed the revision, and thank you for your efforts to address the reviewers' points. We find that your revisions adequately addressed the points of concern. However, since we were all confused by the apparent discrepancy between Fig. 1G and the supplemental data, is it possible to either display the supplemental data differently or to walk the reader carefully through the transition from the supplemental data to Fig. 1G? Your explanation is addressed to the reviewer, but changes in the manuscript for the reader are not apparent. We would be happy to publish your paper in JCB pending this minor change and pending final revisions necessary to meet our formatting guidelines (see details below).

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Suggested eTOC: edited to meet our style requirements:
Maton et al find that human cells re-entering the cell cycle from quiescence have an impaired p53-dependent DNA replication origin licensing checkpoint and slow origin licensing. This combination makes every first S phase under-licensed and hypersensitive to replication stress.

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